Development of PEC-clay nanocomposite for hemorrhage control

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Development of PEC-clay nanocomposite for hemorrhage control

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Master of Technology

in

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(*Specialization*: *Biomedical Engineering*)

by

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(Roll Number: 215BM1002)

based on research carried out

under the supervision of

Prof. Devendra Verma

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May 29, 2017

Supervisor's Certificate

This is to certify that the work presented in the research project entitled "*Development of PEC-clay nanocomposite for hemorrhage control*"submitted by *Sourav Mishra*, Roll Number 215BM1002, is a record of original research carried out by him under my supervision and guidance in partial fulfillment of the requirements of the degree of *Master of Technology* in *Biomedical Engineering*. Neither this thesis nor any part of it has been submitted earlier for any degree or diploma to any institute or university in India or abroad.

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Devendra Verma

Dedication

This thesis is dedicated to my parents,

my brother and
my supervisor for making me who I am.

Signature

Declaration of Originality

I, *Sourav Mishra*, Roll Number *215BM1002* hereby declare that this dissertation entitled *Development of PEC-clay nanocomposite for hemorrhage control* presents my original work carried out as a postgraduate student of NIT Rourkela and, to the best of my knowledge, contains no material previously published or written by another person, nor any material presented by me for the award of any degree or diploma of NIT Rourkela or any other institution. Any contribution made to this research by others, with whom I have worked at NIT Rourkela or elsewhere, is explicitly acknowledged in the dissertation. Works of other authors cited in this dissertation have been duly acknowledged under the sections "Reference" or "Bibliography". I have also submitted my original research records to the scrutiny committee for evaluation of my dissertation.

I am fully aware that in case of any non-compliance detected in future, the Senate of NIT Rourkela may withdraw the degree awarded to me on the basis of the present dissertation.

May 29, 2017
NIT Rourkela

Sourav Mishra

Acknowledgment

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ABSTRACT

External wounds such as cuts, abrasions, lacerations and in cases deep wounds like gunshot wound or even burns are included in common causes of death as they lead to heavy blood loss from human body. Prevention for these issues are use of hemostatic agent in the preliminary stage thereby impeding blood loss for 30 minutes to 1 hour at least and alleviate hypovolemic shock induced deaths in humans. Numerous wound dressing materials have been developed in last few decades which use simple , readily available biomaterials to prepare hemostatic agents either in the form of powder, or gauge or sponge material or beads etc. Almost all commercially available agents are found to have excellent desired characteristics as a wound dressing even though almost each of them lack some major characteristics. For a dressing to be ideal it should be highly hemocompatible, it should have antimicrobial properties, it should be hydrophilic, should have moisture content, should be able to remove easily, should be flexible to use in all types of wounds, not just superficial, flat wounds and lastly it should absorb all the exudates.

Some of the commercially available wound dressing agents are QuickClot, Hemcon Chitogauge, Cel-X etc. QuickClot (Z-Medica)is composed of mineral zeolite powder with a 3D honeycomb structure that allows it to sift molecules by size. When comes in contact with blood, it absorbs water molecules increasing the clotting factors and cell concentrations at the wound. This readily forms clot at the wounded site. However QuickClot was found to have a severe side effect. It appeared to be exothermic and caused local tissue burn at the wounded site. Also use of granular form QuickClot was difficult. Once clot is formed, the powder could only be extracted by surgery. Hemcon , made up of chitosan is a stiff dressing and thus is limited only to superficial wounds. For deep wounds more advanced ChitoFlex and ChitoGauge are used.

Keywords*: *Polyelectrolyte complex*; *Bentonite*; *Wound dressing*; *Hemorrhage

Contents

List of Figures

List of Tables

Chapter 1

Introduction

1.1 Wound healing process in human

1.1.1 Wound

An injury or damage to the integrity of skin, muscle, bone or any tissue of the body by physical means which causes disruption to the normal continuity of structure can be termed as a wound. They can be categorized into abrasion, avulsions, contusions, crush wounds, cuts, lacerations, deep wounds/ velocity wounds and punctures. Depending on the severity they may cause infection, inflammation or even scar formation.

1.1.2 Wound healing

Wound healing is a spontaneous action by the body to counteract the effects of an injury and maintain the integrity of a conventional structure. In this complex and dynamic process, devitalized cellular structures get replaced, and tissue layers regrowth occurs. The process can be one of the two ways. Healing by primary intention or healing by secondary intention, which depending upon the intensity of wound. A small puncture or cut can heal completely within two or three days. However, in the case of severe injuries, it takes time for the body to heal properly. The complex series of events include chemotaxis, phagocytosis, neocollagenesis, collagen degradation, collagen remodelling, angiogenesis, epithelization, and the production of new glycosaminoglycans (GAGs) and proteoglycans.

The overall wound healing process can be described under 4 distinct phases.

- Hemostatic phase
- Inflammatory phase
- Proliferation phase
- Maturation phase

The first phase can be regarded as human body's natural response to the wound. A wound usually disrupts the blood vessels and further response from the body starts

with vasoconstriction followed by a series of complex biochemical reactions called as haemostasis. Here platelets adhere to the wounded site and release cytokines, chemokines, and hormones [1]. Epinephrine, norepinephrine, prostaglandins, serotonin, and thromboxane are called vasoactive mediators and cause blanching of the wound temporarily. Platelet activation leads to the release of chemokines whose function is to attract inflammatory cells to the area. This process leads to the next phase in the healing process. Studies show that a defective formation of clot such as the absence of fibrin stabilising factor in clot affects wound healing [2].

After the completion of haemostasis, blood vessels dilate, through a process known as vasodilation which is guarded by some hormonal changes in the body. This allows cells, antibodies, growth factors, nutrients, etc. to reach the wounded area and accelerate the healing. This vasodilation is mediated by histamine, prostaglandins, kinins, and leukotrienes. However, this leads to the excessive deposition of exudates at the site of injury. Following signs of maceration needs to be monitored. The characteristic signs of inflammation like erythema, heat, edema, pain can be seen and felt during this period. The phagocytic cells; neutrophils, polymorphonucleocytes and local mast cells are the predominantly work in this phase. Any devitalised necrotic tissues are autolysed as a host response effect. They provide the first line of defence against any infection. Broken fibrin is cleaned up as and along with the degradation product.

During proliferation phase, the wounded site is restructured at a cellular level with new tissue consisting of collagen and extracellular matrix. Organisation of a new network of blood vessels develop. The process is called angiogenesis. Healthy granulation starts on the epithelial layers. However, the rate of granulation highly depends upon the fibroblasts and availability of sufficient oxygen and nutrients. Healthy granulation tissue is an amorphous structure, granular and uneven in texture providing the restriction to bleeding. A granulation pink or red in appearance indicates a healthy granulation tissue whereas dark granulation tissue can be indicative of ischaemia or poor perfusion. Epithelial cells finally resurface the wound.

Maturation is the final phase. It begins after the wound closer. Type III collagen is remodelled into type I collagen. Cellular activities decrease at the wound site and the number of blood vessels in the wounded area regress and decrease.

A complete but abnormal wound healing can be characterized by the production of an exuberance of fibroblastic proliferation which can result in hypertrophic scar or keloid formation. Insufficient wound healing may result in hypotrophic or atrophic scar formation.

From the above discussion, it is evident that skin is regarded as the first line of defence followed by blood. The blood with its unique chemistry and constituent cells provides a strong line of defence against microorganisms and maintain regular activities.

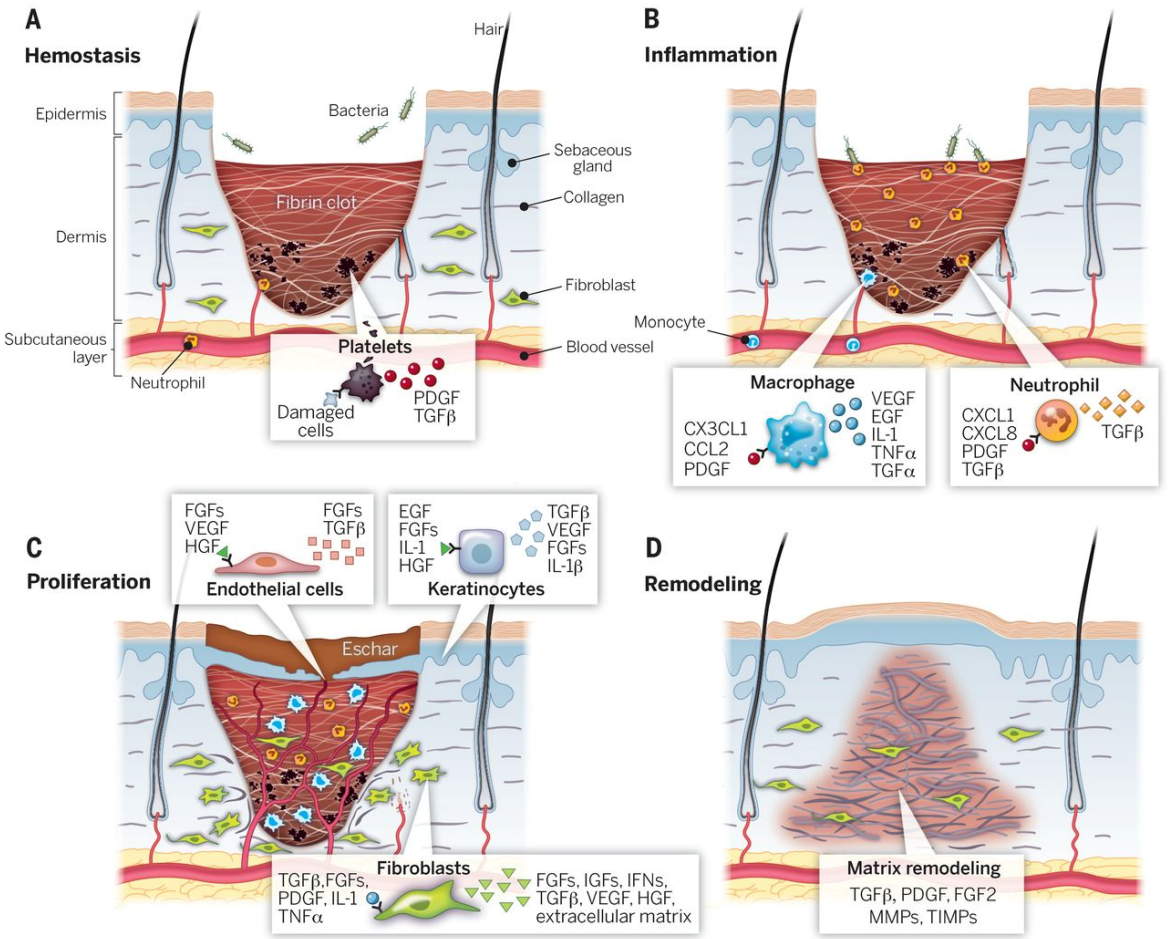


Figure 1.1: Stages of wound healing *(source:https://www.pinterest.com/pin/)*

1.1.3 Blood cells and other wound healing mediators

Blood is a specialized body fluid which transports micro and macro nutrients, gases and other metabolites needed for life sustaining, either dissolved in plasma or through conjugation with protein entities present in human body. Blood vessel endothelium has some important regulation mechanism to impart along with functional defence of blood and its cells. The vascular endothelium secretes locally acting messengers such as prostaglandins, nitric oxide (NO) and peptides CNP and endothelin-1. They maintain vascular homeostasis and influence processes like blood pressure, cell proliferation etc. Prostaglandins are involved with clotting. Prostacyclin (PGI_2) has anticoagulant properties. PGI_2 along with nitric oxide, NO (one of the EDRFs) acts as vasodilator and are used in second stage of wound healing. Thromboxane (TXA_2) is a procoagulant and is derived from platelets. NO-induced production of cyclic GMP is responsible for:

- Vasorelaxation (the EDRF effect) in vascular smooth muscle cells (VSMC)
- Inhibition of proliferation of VSMC and endothelial cells
- Inhibition of platelet adhesion and aggregation on endothelium

- Inhibition of endothelial apoptosis (at low NO concentration)

In general, blood is composed of plasma, blood cells, and different micro and macromolecules including proteins. Blood plasma is almost 92% water by volume and constitutes almost 55% of blood volume. It carries different proteins which help clotting as secondary defence mechanism. The cells present in blood are:

- Erythrocytes
- Thrombocytes
- Leukocytes
  - Granulocytes
    - Eosinophil
    - Basophil
    - Neutrophil
  - Agranulocytes
    - Monocytes
    - Lymphocytes

Erythrocytes

During dermal wound healing erythrocytes are believed to affect the extracellular matrix protein expression of dermal fibroblasts. Some studies also include the hypothesis of dermal fibroblast matrix metalloproteinase (MMP)-1 level being affected by RBCs. MMPs have role in ECM remodelling.

Through the studies of Greiling et al. a relation has been established between fibronectin production and re-epithelialization of wounded site. Fibronectin in conjunction with PDGFs promote the expression of appropriate receptors. The migration of the former from the periwound stroma into the fibrin clot for the initiation of the re-epithelialization process is followed by signalling to fibroblasts and keratinocytes [3]. Hence, RBC has a passive effect on re-epithelialization as post wound healing procedure by the upregulation of fibronectin.

Thrombocytes

In normal flowing condition platelets are biconvex, although when treated with EDTA they appear somewhat spherical. Under certain conditions like rupture to wall vessel or damaged tissue condition, stored blood platelets migrate from the spleen to the peripheral blood. They change their surface structure and characteristics, become irregular and sticky, extending pseudopods. They adhere to nearby surfaces or form clump adhering to each other. The platelet glycocalyx is thicker than cell membranes of leukocytes and erythrocytes

being 20-30 nm in thickness. This thick layer possess high adherence property thereby responds readily to hemostatic demands. The platelet surface charge is negative. It carries its functional environment with it, meanwhile repels other platelets, other blood cells, and the cells of blood endothelium all with similar negative charge surface. The plasma membrane protrude inward towards the platelet interior and produce its unique surface-connected canalicular system (SCCS), which are twisted throughout the platelets and store proteins used in abnormal hemorrhagic condition. Dense tubular system (DTS), a remnant of the rough endoplasmic reticulum, lay parallel to the glycocalyx membrane. The DTS sequesters Ca^{2+} and bears carries enzymes like phospholipase A_2, cyclooxygenase, and thromboxane synthetase which are involved in platelet activation process. The discoid shape of platelet is maintained by a circumferential bundle of microtubules. The later get reassembled in long parallel bundles forming pseudopods during platelet shape change to provide rigidity.

There are mainly two types of secretion vesicles in platelets. The dense granules and the α-granules. The α-granules are filled with proteins, VWF, thrombin and certain growth factors. Upon platelet activation α- granule membranes fuse with the SCCS. Proteins from α- granules flow to the nearby microenvironment. There they participate in platelet adhesion and aggregation and support plasma coagulation. Along with 50-80 numbers of α-granules there dense bodies otherwise called dense granules present 2 – 7 in number, per platelet.

Basophil

Basophils contain heparin, an anticoagulant which acts on Factor X and prevents blood from clotting too quickly by inhibiting prothrombin to thrombin conversion. They also contain the vasodilator histamine, promotes blood flow to tissues.

Eosinophils

These are regarded as source of cytokines transforming growth factor, TGF-and TGF-along with macrophages, neutrophils, keratinocytes, fibroblasts, and endothelial cells [4].

Neutrophils

Neutrophils being the first immune cell types to reach the wound bed through migration are activated by microbial or inflammatory stimuli. The activation of neutrophils causes the release cell-free DNA, histones, and antimicrobial proteins. Cell-free DNA (CFDNA) activates the intrinsic pathway of coagulation. Histones released in the process take part in platelet-dependent mechanisms to promote thrombin generation [5].

Monocyte

These are the second immune cell type to migrate towards wound bed. The process is facilitated by monocyte chemoattractant protein-1 (MCP-1) which help the migration as well

as a transformation of the monocytes cells into macrophages. The later disposes of the debris and neutrophils through phagocytosis.

1.1.4 Mechanism of Coagulation

Severe blood loss from ruptured blood vessels is called hemorrhage. The body has its complex and coordinated mechanism of controlling hemorrhage, known as hemostasis. Hemostasis involves vasoconstriction and forms the clot at the injured site. The process starts from the activation of platelets at the site of injury to complete healing of the tissue. Damaged endothelium at injured site release Endothelium Derived Contraction Factors (EDCFs), some unique peptide endothelin, leading to vasoconstriction. This limits the flow of blood to the wound bed.

Platelet activation

A break in endothelium, exposes the collagen underneath. Platelets get adhered to collagen exposed from damaged endothelium by an von Willebrand factor (vWF) mediated reaction. The vWF connects platelet surface to collagen fibrils by forming a bridge between a specific glycoprotein complex on the surface of platelets (GPIb-GPIX-GPV) and collagen fibrils. Also vWF stabilizes coagulation factor VIII in normal circulation by binding to the active site.

Platelets adhere to one another through fibrinogen present already in blood and to collagen under the endothelium through vWF, forming a platelet plug. On the surface of the activated platelet are glycoprotein IIb/IIIa and VWF receptors. Which provides the site of adherence to fibrinogen. The binding of fibrinogen by glycoprotein IIb/IIa on two platelets causes platelets to adhere to each other. Activated platelets form pseudopods which help them adhere to one another and to wound surface. A little of the fibrinogen is released from the alpha granules also.

The release of intracellular Ca^{2+}, activates myosin light chain kinase (MLCK) enzyme. Formation of pseudopods is facilitated by activation of MLCK, which then phosphorylates the light chain of myosin. The later then interacts with actin protein in platelet cytoskeleton, resulting in altered platelet morphology and motility. Thus as the platelet is activated, its shape changes to an amorphous form with pseudopods. Further process after platelet activation can be explained in two pathways,

- The extrinsic pathway
- The Intrinsic pathway.

Both are initiated by distinct mechanisms and converge to lead the common pathway finally producing a fibrin clot.

Intrinsic pathway

Intrinsic pathway starts from contact phase which is initiated by interaction with the phospholipids, primarily phosphatidylethanolamine (PE) of circulating lipoprotein particles such as chylomicrons, VLDLs, and oxidized LDLs, when pre-kallikrein (PK), high molecular weight kininogen (HMWK), Factor XI and factor XII are exposed to a negatively charged surface. Contact activation can be initiated on bacterial surface as well. Urate crystals, fatty acids, protoporphyrin, amyloid β, and homocysteine can interact with blood to initiate contact activation.

After the contact phase components get adhered to potentially negative charged surface (usually platelet plug), prekallikrein is converted to kallikrein. The later then activates the factor XII (Hageman factor), an endopeptidase enzyme to factor XIIa. Factor XIIa has two contributions in clotting cascade. It activates Factor XI (Plasma thromboplastin antecedant) into factor Xia. It establishes a reciprocal activation cascade by hydrolyzing more prekallikrein into kallikrein.

Factor XIa activates factor IX to factor IXa in presence of Ca^{2+} ion. Factor IX is a proenzyme and contains vitamin K-dependent γ-carboxyglutamate (gla) residues. Its glutamate residues binds to Ca^{2+} ions, serene protease activity is activated in result of that. Factor II, VII, IX, and X are also gla-containing proenzymes. Activated factor IXa activates factor Xa from factor X cleaving an internal arg-ile bond.

The formation of tenase complex is facilitated by presentation of phosphatidylserine (PS) and phosphatidylinositol (PI) on their surfaces platelet membrane surface, which allows the comprising Ca^{2+} ion and factors VIIIa, IXa and X. This activates factor Xa.

Factor VIII in this process acts as a receptor in activated form (VIIIa) providing binding site for factors IXa and X. The presence of minute quantity of thrombin in circulation activates factor VIII to factor VIIIa (the actual receptor). However the concentration of thrombin establishes a feedback mechanism, which controls the extent of coagulation. As the concentration of thrombin increases above a threshold, it cleaves factor VIIIa thus inactivating it and controlling the extent of tenase complex formation as well.

Extrinsic Pathway

In case of external cut, bruise or any kind of external tissue injury, a localised transmembrane receptor called Tissue factor is released by local tissue, which initiates extrinsic pathway as an exogenous agent and thus the name. Tissue factor, also known as factor III is a cofactor in the activation of factor X reaction mediated by factor VIIa. It is usually expressed by the cells surrounding blood vessels. Trace amount of it are found in blood circulation, some in liver and heart as well. It was found that TF present around blood vessels is found in complex state bound with FVII, and gains individual form after injury [6].

Factor VIIa is a gla residue containing serine protease enzyme. It carries the

activation reaction of factor X to factor Xa, with the help of TF. The extrinsic pathway is controlled by a complex formed by TF and factor VIIa-Ca^{2+}-Xa complex. The protein, lipoprotein-associated coagulation inhibitor (LACI). . LACI is also known as anticonvertin binds to this complex specifically inhibiting the extrinsic pathway. It is composed of 3 tandem protease inhibitor domains and forms a feedback loop controlling the extent of extrinsic pathway. Domain 1 binds to factor Xa and domain 2 binds to factor VIIa in presence of Xa.

The common pathway

Activation of Factor X to factor Xa is the common point of both intrinsic and extrinsic pathway and marks the beginning of common pathway.. Factor Xa activates factor II to factor IIa, thrombin. Thrombin facilitates the conversion of fibrinogen to fibrin. The activated surface platelets play host to the prothrombinase complex formation where the activation of thrombin occurs. This complex consists of the platelet phospholipids (phosphatidylinositol and phosphatidylserine), Ca^{2+} ion, factors Va and Xa, and prothrombin. Factor V has similar as that of factor VIII in tenase complex formation. It works as a cofactor in the prothrombinase complex formation. Low concentration of thrombin facilitates the activation of factor V (analogous to Factor VIII) and is inactivated by increased levels of thrombin, working a feedback loop again.

Fibrinogen (factor I) in its inactivated normal form, forms a bridge between platelets by binding to their GpIIb/IIIa surface membrane proteins during platelet activation process. It is a hexamer and consists of 3 pairs of polypeptides ([Aα][Bβ][γ])2. The 3 pair polypeptide chains are covalently linked at their N-terminals through disulfide bonds. The former portions of the Aα and Bβ chains (the A and B component) comprise the fibrinopeptides, A and B, respectively. Thrombin activates the release of the fibrinopeptides forms fibrin monomeric units (αβγ)2. These monomers form a mesh like structure, spontaneously aggregate to form a weak fibrin clot. In addition to fibrin activation, other functions of thrombin involve convertion of factor XIII to factor XIIIa, This is a transglutaminase and forms cross-linking bridges composed of covalent bonds between the amide nitrogen of glutamines and ε-amino group of lysines in the fibrin monomers to make a strong clot.

1.1.5 Ideal dressing

An ideal dressing should be an efficient barrier to bacterial infection; it should be able to maintain moisture environment around the wound bed, allow the gaseous exchange, and be able to remove exudates to maintain a clean microenvironment. It should also be non-allergenic, non-adherent, non-toxic, hemocompatible and when necessary should be able to be removed easily without causing pain, the biomaterial should be readily available and should require minimal processing, possess antimicrobial properties and promotes

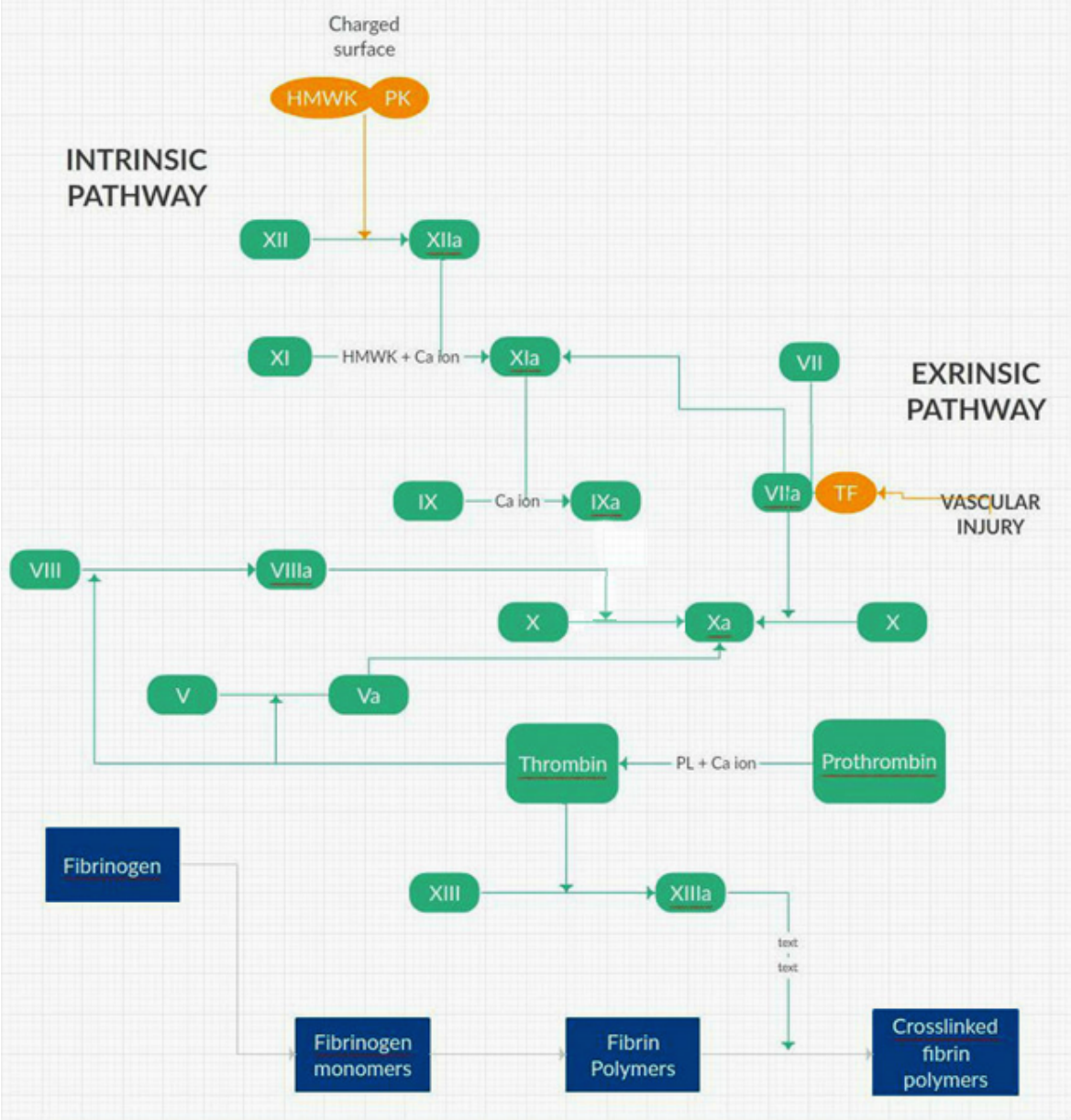


Figure 1.2: Clotting Cascade

wound healing.

1.1.6 Wound dressing material

History of wound dressing materials can be dated back to 1600 BC when plastic covered Linen strips soaked in grease was used to occlude wounds. There has been evidence of use of clay tablets by people from mesopotamian origin during 2500 BC. Hippocrates of Greece used wool as bandage in boiling form, in water or alcohol [7]. Proper standardised wound care began to establish during 20th century. In 1891 use of cotton gauge was established whcich was in proper woven form with absorbant properties.

The following table is a summerized form of review of wound dressing materials [8].

Table 1.1: Comercial wound dressing samples and their uses

Form of dressing	Use	Example
Semipermeable	Superficial wound. Shallow wound with low exudates	Opsite™, Tegaderm™
Semi-permeable foam	Moderate to highly exudating wounds, granulating wounds. Used in lower leg ulcers.	Lyofoam™, Allevyn™
Hydrogels dressing	Dry chronic wounds, necrotic wounds. ulcers and burn wounds.	Intrasite™, Nu-gel™
Hydrocolloid dressing	Moderately exudating wounds, pressure sores, paediatric wound, minor burn wounds, traumatic wounds,	Granuflex™, Comfeel™
Alginate dressing	Moderate to heavy drainage wounds	Sorbsan™, Kaltostat™
Biological	Suitable for diabetic foot ulcer and venous leg ulcer.	Laserskin™, Biobrane ™
Medicated dressings	Cleaning or debriding agents for necrotic tissue, antimicrobials	Cutisorb™, DebridaceTM

Some of the commercially available wound dressing agents are QuickClot, Hemcon Chitogauge, Cel-X etc. QuikClot from Z-Medica is a clay based bandage composed of mineral zeolite powder with a 3D honeycomb structure that allows it to shift molecules by size. When comes in contact with blood, when in aqueous medium it absorbs water molecules thereby increasing the concentration of clotting factors blood cells at the wound site. This readily forms clot. However Quickclot was found to have a severe side effect. The reaction of it with blood is apprered to be exothermic and caused local tissue burn at the wounded site [9]. Also use of granular form QuickClot was difficult. Once clot is formed, the powder could only be extracted by surgery.

Hemcon , made up of chitosan is a stiff dressing and thus is limited only to superficial wounds. For deep wounds more advanced ChitoFlex and ChitoGauge are used. Celox being another dressing material in powder form is good for superficial wounds, but when used in deep wounds , it may get carried away with the flow of blood and adhere somewhere inside blood vessels forming clot [10].

Chapter 2

Litrature Review

Chitin was discovered in 1811 in France by prof Henri Braconnot and it was of fungal origin. The name chitin comes in 1830s retrieved from the Greek etymology. It means “A Coat Of Mail”. Later it was found in abundance from shells of shrimps, prawns and other crustaceans.

Chitosan was discovered in 1859 by prof C. Rouget. It is an alkaline deacetylation product of chitin. The degree of deacetylation varies considerably and affects structural and chemical properties of chitosan. The degree of deacetylation being close to 0% or 100% yields a products promoting increased cell adhesion and decreased rate of degradation which was not in the case of intermediate deacetylation [11]. Even being the second most abundant natural polysaccharide, chitin has found very little usage due to its inertness. Chitosan is a copolymer of two units names as (1 → 4)-2-acetamido-2-deoxy-β-D-glucan (N-acetyl D-glucosamine) and (1 → 4)-2-amino-2-deoxy-β-D-glucan (D-glucosamine) [12]. The -OH function of glucopyranose ring in chitosan are susceptible to nucleophilic attack. Typically, the molecular weight of commercial chitosan has a varied range from 3.8-20 kDa.

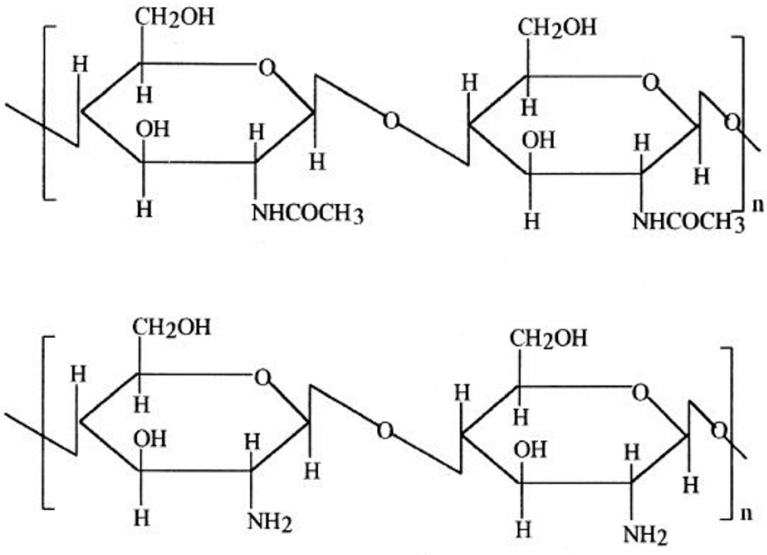


Figure 2.1: Structure of chitin (i) and chitosan(ii)

The degree of acetylation determines the density of the amino groups in chitosan

macromolecule. They behave as the cationic group as they can carry an extra positive charge which is pH dependent, the later being the cause of degree of protonation. At high degree of deacetylation chitosan exhibits an extended form and flexible polymeric chain whereas at lower degree of deacetylation it adopts a more coiled rod like structure [13]

Chitosan containing a large number of amino groups in its structure which in fact imparts to its cationic behavior also exhibits a pH-sensitive behavior as a weak polybases. Chitosan dissolves frequently at low pH while at higher pH range it is almost impossible to dissolve. Chitosan containing a large number of amino groups in its structure which in fact imparts to its cationic behavior also exhibits a pH-sensitive character as a weak polybases . Chitosan dissolves frequently at low pH while at higher pH range it is almost impossible to dissolve. The protonation of amine groups in chitosan at only less pH conditions drives the pH sensitive swelling properties. This protonation is followed by chain repulsion, diffusion of H+ and opposite ions together with water inside the gel and dissociation of secondary interactions. Preparation of chitosan hydrogels followed by scaffolds by the formation of polyelectrolyte complexation substitutes effectively, the covalent cross-linking hydrogels. PECs are usually biocompatible and exhibit good swelling behavior.

Chitosan provides a matrix for 3 dimensional tissue growth and activates macrophages for tumoricidal activity [14]. Being a hemostat, it helps in conventional blood clotting and blocks nerve endings and hence brings relief to at the injured site [15]. Cell proliferation and tissue architectural onset is pivoted by chitosan [16]. It thereby depolymerizes to release N-acetyl- β -D-glucosamine. This onsets proliferation of fibroblast and helps in collagen deposition ot the site in order, also stimulates increased level of natural hyaluronic acid synthesis at the wound site. It helps in increased rate of wound healing and scar prevention.

The inherent polycationic nature of chitosan makes it a potential analgesic material [17]. The amino group ($-NH_2$) along D-glucosamine residues are protonated at the site of inflammation causing a relief of pain.

Chitosan being a natural polysaccharide bears covalent glycosidic bonds which are breakable. Several proteases and mainly lysozyme degrade chitosan into variable length oligosaccharides which are incorporated in normal metabolic activity of or can be excreted [11].

In the early phase of wound healing, infiltrating polymorphonuclear leukocytes (PMN) clean foreign agents in the wound area, while chitosan accelerates the infiltration of PMN, consequently accelerating wound cleaning [18].

Positively charged chitosan surface can electrostatically attract the neuraminic acid residues of RBC surface glycoprotein. Erythrocytes lose their typical biconcave shape indicating their procoagulant activity. Further negatively charged blood proteins like fibrinogen electrostatically attach to chitosan surface initiating platelet adhesion and activation.

Chitosan has been found to have higher activated partial thromboplastin time (aptt time)

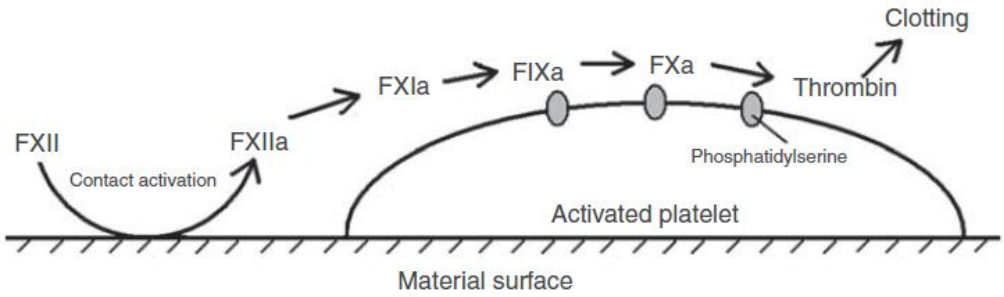


Figure 2.2: Schematic representation of platelet activation on material surface [19]

showing a little delay in onset of intrinsic mechanism of blood clotting. This effect has been attributed to a phenomenon called the "adsorption-dilution effect" which passively delays the clotting time [19].

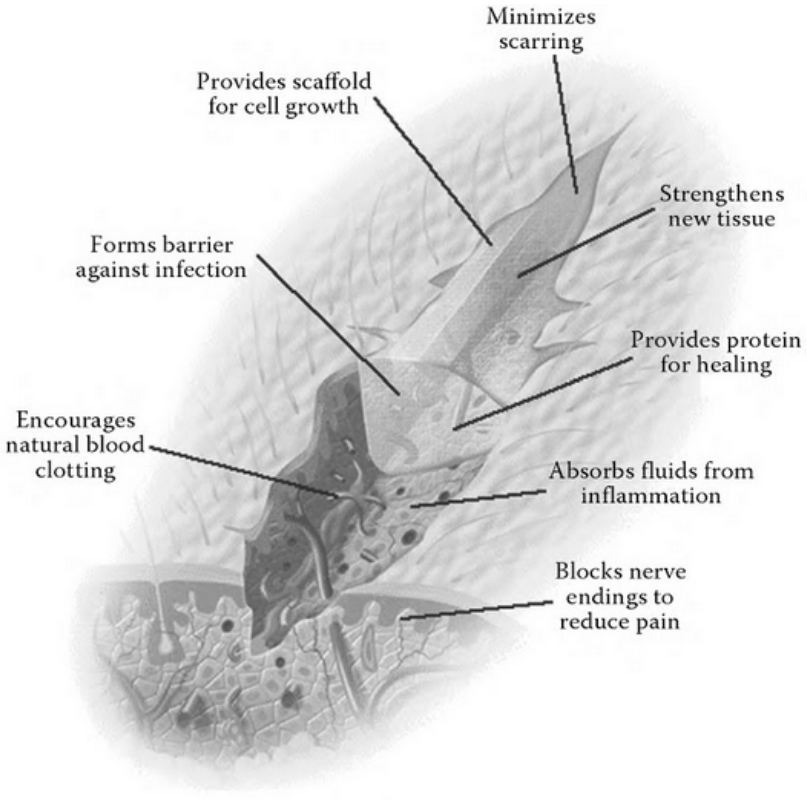


Figure 2.3: Schematic representation of benifits of chitosan on wound healing [20]

Alginate is a natural polysaccharide [21]. It is extracted from marine brown algae. Alginate is copolymer with linear structure that consists of two polymeric links, i.e. (1-4)-linked β -D-mannuronic acid (M) and a-L-guluronic acid (G) residues [22]. The two monomeric units are of uronic acids and their relative concentration vary along the polymer chain along with their arrangement, depending on the origin of the alginate. Alginate wound dressings possess most of the desired properties of ideal wound care material. Hygroscopy, bactericidal property at wounded site, and help in wound healing. Calcium alginate has been found to have profound effect as a wound dressing material. Even there are commercialised

alginate based products available in market for wound care and management. Calcium alginate dressings have been found to provide a significant healing effect in STSGs (split thickness skin graft) donor sites [23]. The pain severity significantly reduces in burn patients. Its ease of care has been a great advantage for nursing personnel. The combined use of calcium sodium alginate and a bio-occlusive membrane dressing with calcium alginate eliminates the problem of seroma formation in the management of split skin graft donor sites. Thomas et al. [24] have reported that alginates accelerate healing process by activating macrophages to produce TNF-α which initiates inflammatory signals.

Polyelectrolytes are macromolecules which exhibit a net positive or negative charge when dissolved in a polar solvent like water. When opposite charged polyelectrolytes come into contact with each other , form polyelectrolyte complex or otherwise known as polysalts. As the cationic and anionic polyelectrolyte get mixed, the reaction creates an exotherm as entropy of the system shifts from ideal [25].

Chitosan and Alginate (both polyacids) being cationic and anionic in nature form such polyelectrolyte complex. The resulting material is superior to both the individual ones in strength and in some properties and provide optimal support to wound care. Studies on chitosan, alginate polyelectrolyte complex have shown to have significant impact on wound healing, promoting platelet adhesion and thereby coagulation in case of deep wounds and burns as well.

Bentonite predominantly consists of montmorillonite: a clay mineral belonging to a class of phyllosilicates called smectites. Bentonites may contain a variety of accessory minerals in addition to montmorillonite.Its density when dry varies depending on the quality, and may range from 2.2 to 2.8 g/cm3. The charge deficiencies of montmorillonite is described by extensive isomorphous substitution for silicon and aluminium by other cations. Hydrated water molecule and cations coexist between the unit layers and basal spacing varies from few angstrom to infinity (after swelling). Several wound healing product like quikclot have been developed using clay minerals like zeolite and are found to have better effect on dressing material. QuickClot having zeolite releases Ca2+ ion into blood and thereby causes improved clotting [26].

Chapter 3

PEC samples

3.1 Materials and Methods

Cell culture tasted Chitosan with degree of deacetylation greater than 90% and molecular biology grade sodium alginate were obtained from HiMedia. Bentonite powder (Aluminium Silicate hydrate) is obtained from Fisher scientific. The test results were compared with HemCon ChitoGauze® PRO and Quikclot advanced clotting sponge. PT (Thromborel® S) and APTT (Actin FSL) reagents were obtained from Siemens Healthcare.

3.1.1 Preparation of PEC Dressing

Chitosan solution (1% W/V) was prepared in water. Hydrochloric acid (HCl) was added dropwise to maintain acidic pH. Sodium alginate was dissolved in water. pH of chitosan solution was maintained around 5. Sodium alginate solution prepared has an acidic pH as well (pH 6-6.5). Chitosan solution was taken in a syringe and dropwise added to alginate solution. Beads are formed in alginate base. Ultrasonification of these samples were done to prepare an uniform mixture which is the resultant PEC solution. The composition of the PEC were varied starting from 70% chitosan-30% alginate with 10% decrement in chitosan and 10% increment in alginate concentration up to 30% chitosan-70% alginate. These solutions were frozen at -200 C for 12 hours. Then the samples were lyophilised at -1200 C in a lyophilizer. Resultant scaffold produced is the desired material to be tested. Scaffolds of pure chitosan and alginate were also produced. For characterisation studies, Environmental Scanning Electron Microscope (E-SEM), study was carried out.

3.1.2 Hemolysis Assay

Hemolysis assay was taken from Mallick et al. [27]. 0.9% NaCl solution was prepared by diluting common salt in water. This is known as physiological saline. Blood is diluted with physiological saline in the ratio of 4:5. The resulting solution forms the stock solution. The dressings were cut into equal sizes (12 mg each). The dressings were placed into polypropylene tubes and 0.5 mL of diluted blood was dispensed on them. Two different

control (positive and negative) were also made. Same amount of blood was added to both control, while .01M HCl was added to the positive control rupturing the RBC fraction. All the tubes were diluted with physiological saline by similar amount. The tubes were centrifuged at 2000g for 20 minutes. OD of the supernatants was taken at 540 nm.

$$Hemolysis\% = \frac{OD_{test} - OD_{-ve}}{OD_{+ve} - OD_{-ve}} \times 100 \tag{3.1}$$

Samples having hemolysis percentage <5% are highly hemocompatible, 5-20% are semi hemocompatible and samples having hemolysis percentage above 20

3.1.3 Blood Clotting Assay

The whole blood clotting assay was taken from Shih et al. [28] and was modified. The dressings were placed into 50 mL polypropylene tubes and pre-warmed to 37ºC. 200 µL of anticoagulated whole blood was then dispensed onto the dressings. 20 µL of 0.2 M calcium chloride was added to initiate coagulation. After 10 min incubation at 37ºC and 30 rpm, the RBCs that were not trapped in the clot were hemolysed by pouring 25 mL distilled water slowly along the walls of the tube. This was done in an interval of 5, 10, 15, 20 minutes. The absorbance (OD) of the resulting hemoglobin solution was taken at 540 nm.

3.1.4 PT test

This test represents the duration in which the extrinsic pathway takes place. A prolonged PT time may signify an enhanced anticoagulant activity or chemical change to factor VII, X, V, II, I (fibrinogen). Another value INR is also associated with PT test. INR value greater than 1 shows delayed clotting. A typical range of INR for person not taking blood thinner medication is 2 to 3.5.

PT test of the sample dressing were done at CWS hospital Rourkela. Anticoagulated whole blood was collected and centrifuged at 4000g to isolate platelet poor plasma (PPP). Now 2 ml of PPP was taken in eppendorf tubes containing dressing material and was incubated at 37ºC for 1 hour.

100ml of sample treated PPP was transferred to test cup and was further incubated at 37ºC for 3 minutes and PT reagent was added 5 seconds prior to the end of incubation. PT reagent was also incubated and kept at 37ºC prior to use.

3.1.5 APTT test

APTT, also known as activated partial thromboplastin time is a medical test characterizing blood coagulation. The APTT test was performed at CWS hospital, Rourkela. Anticoagulated whole blood was collected and centrifuged at 4000g to isolate platelet poor

plasma (PPP). Now 2 ml of PPP was taken in eppendorf tubes containing dressing material and was incubated at 37ºC for 1 hour. 100ml of sample treated PPP was transferred to test cup and was further incubated at 37ºC for 1 minute. 5 second prior to the incubation time finishes, APTT reagent was added and incubated for 3 more minutes. Again calcium chloride was added 5 seconds prior to the end of incubation.

3.1.6 Platelet Isolation and Adhesion

Whole blood was anticoagulated with trisodium citrate before collection. The anticoagulated whole blood was incubated at 37ºC for 15 minutes. Then the blood was collected in 15 ml polypropylene tubes and centrifuged at 800g for 15 minutes to separate blood cells and platelet rich plasma (PRP). About 2 ml of PRP was collected from 15 ml whole blood. The PRP is then mixed with PBS buffer at 1:3 ratio to increase the working volume. Now 100ml of the working solution was poured on the dressing. The dressing is now incubated for 15 minutes. After incubation, dressings were dip rinsed in PBS buffer thrice. This removes the unattached platelets from the dressings. To rupture the attached platelets, dressings were put inside PBS buffer containing 1% TritonX100 surfactant. The surfactant is used for LDH test to determine the percentage of platelets adhered to the dressings. A series of PRP with different dilution was treated with TritonX100 to obtain a platelet dilution curve and was compared to the sample data obtained to measure qualitatively the percentage of platelets adhered.

3.1.7 Swelling Studies

The swelling capacity of the dressings was determined in trisodium citrated whole blood and phosphate-buffered saline (PBS). The pre-weighed dry dressings (wini) were placed into tissue culture wells containing either trisodium citrated whole blood or PBS. The dressings were weighed at an interval of 2 min for 30 min (Wwet). The swelling ratio of the dressings at each time interval was determined by the following formula:

$$Swellingratio = \frac{W_{wet} - W_{initial}}{W_{initial}} \times 100 \tag{3.2}$$

3.2 Results and Discussion

Spontaneous complexation occurs when polymers containing opposite charges are allowed to interact. This complexation happens at the interface of the polymer solutions and is a result of strong electrostatic interaction existing between them. Polyelectrolyte complexes (PECs) so formed do not solubilise in water and precipitate out. Sonication of the solution then produces a homogenous PEC solution

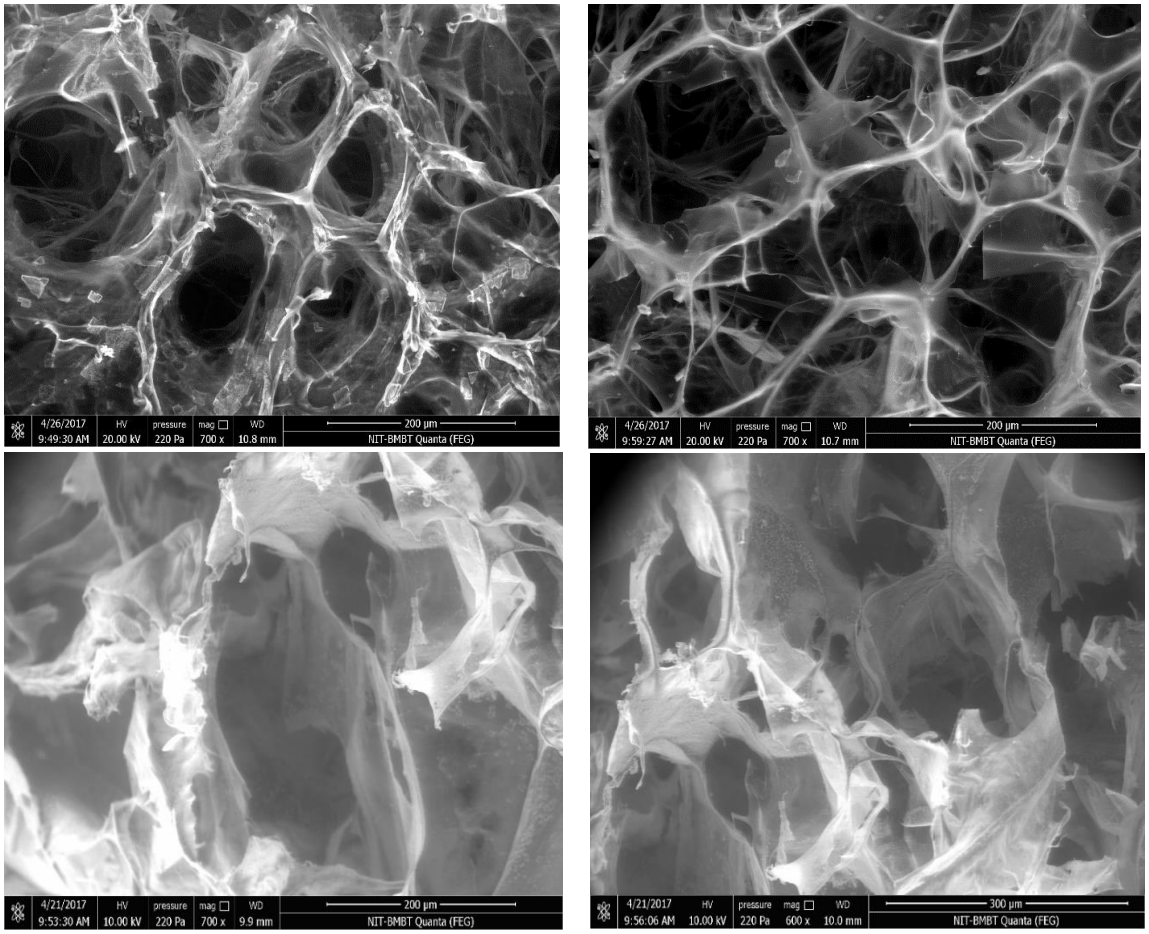


Figure 3.1: E-SEM image of (a) CA64, (b) CA73, (c) AC64 and (d) AC73

Above images are the E-SEM images of sample CA64 and CA73, AC64 and AC73 respectively, taken at a magnification of 700x. The images show highly porous structure. The surface appearance did not vary among CA64 and CA73. Similarly Surface structure of AC64 and AC73 were found to be similar.

3.2.1 Hemolysis Assay

A hemolysis assay depicts the hemocompatibility of a material. Erythrocytes account for almost 40-45% of total blood volume. Any foreign material when exposed to blood from human body likely to cause change in local chemistry or/and concentration of blood. However a minute change in blood environment can cause erythrocytes to rupture and hemoglobin to get released to extracellular environment. Thus , it is essential for a dressing sample to be hemocompatible.

Hemolysis percentage of a sample, if lies above 20% is considered hemolytic. From the aforementioned graph it is evident that pure alginate and chitosan sample show moderate hemolysis and a good hemocompatibility. PEC with higher chitosan concentration

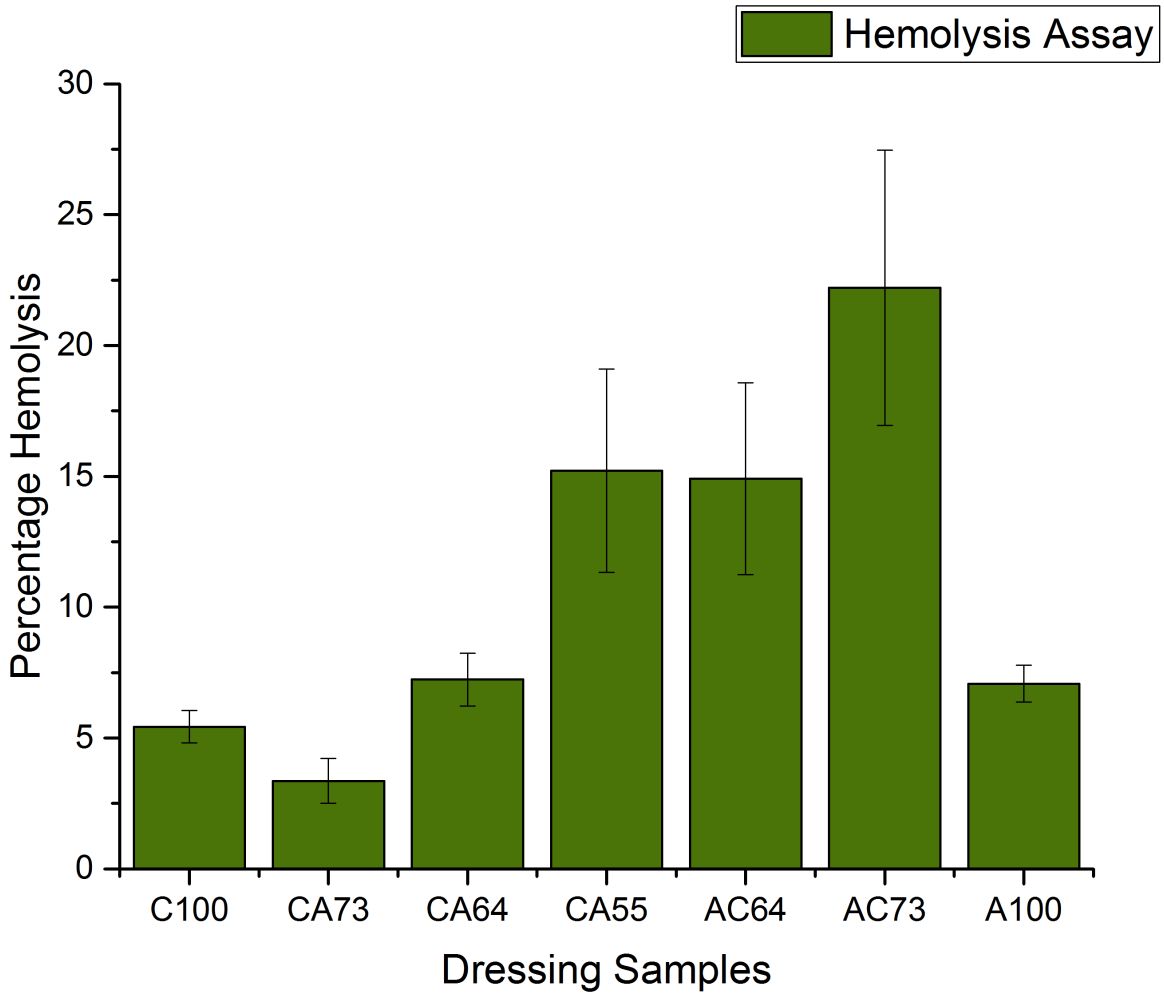


Figure 3.2: Hemolysis Assay graph of PEC samples

have better hemocompatibility and hemocompatibility decreases with decrease in chitosan concentration (increase in alginate concentration as well). It has been known that presence of hydrophilic group renders the material to be less hemolytic [29], which can explain the lesser hemolytic nature of pure chitosan and alginate samples. Same way presence of cationic surface charge promotes the adherence of RBCs among themselves and also decreases the chance of hemolysis [30]. This explains the hemocompatibility of PEC with higher chitosan concentration.

3.2.2 Blood Clotting Assay

The following graph shows the clotting rate of PEC samples.

OD values were measured at an interval of 3, 8, 13 and 18 minutes. The slope of the graph in various interval shows change in OD in between those time intervals and depicts the rate of clotting. In whole blood , as seen from the graph, clotting rate starts to increase after 8 minutes, which is almost similar to sample C100. among PECs with increase in alginate concentration onset of clotting happens earlier. In pure chitosan and sample CA73, steep

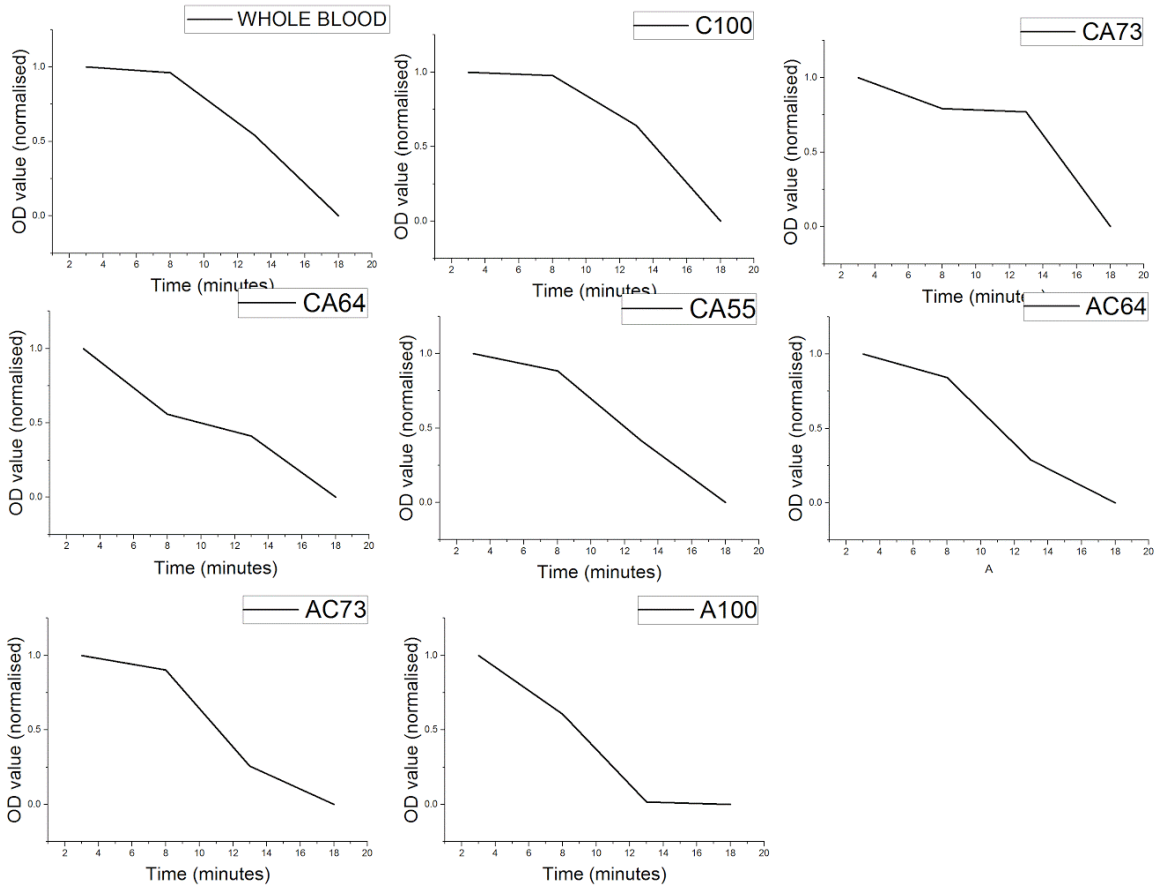


Figure 3.3: Blood clotting Assay graph of PEC samples

slope in interval 13-18 shows , delayed onset of clotting but after onset , clotting becomes faster. Among all , pure alginate sample showed fastest clotting even better than chitosan, even though chitosan is famous for its thrombogenic effect.

3.2.3 PT test

The following graph shows the Prothrombin time, or the time taken for the extrinsic pathway to complete. An abnormal PT time (usually above 24) is usually caused due to anomaly in extrinsic pathway mechanism.

From the graph it is shown that for normal plasma the pt time shows 10 seconds. Scaffold treated plasma delays the pt time maximum 8 seconds. Even though variance is not much, the higher positive charge samples show more delay than lower positive charge samples. Pure alginate showed the least delay.

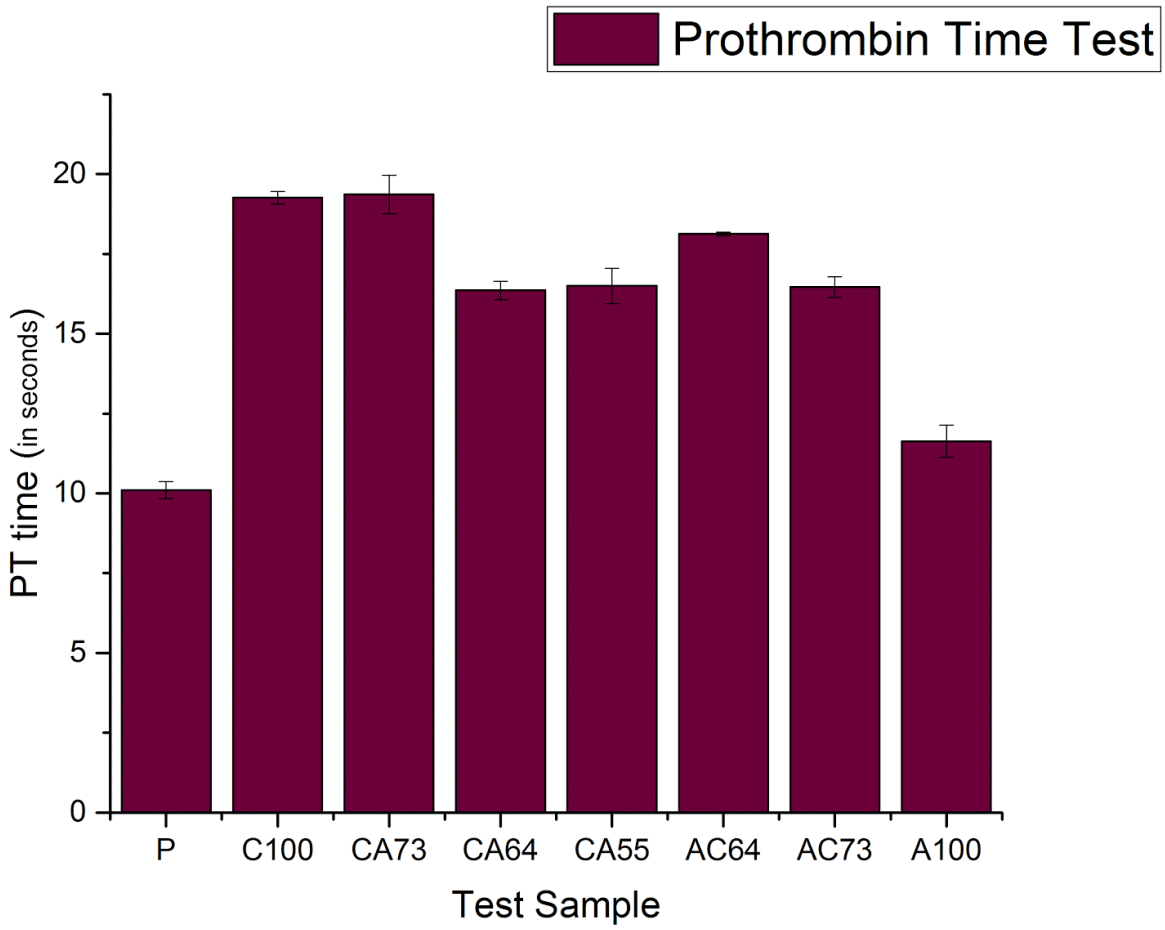


Figure 3.4: PT time test graph of PEC samples

3.2.4 APTT Test

The aforementioned graph shows delayed aptt time with higher chitosan concentration samples. For normal platelet poor plasma, the aptt times vary around 43 seconds. And with higher alginate concentration, aptt time tends to be lower. Even though chitosan is known to be highly thrombogenic, the delayed time may be explained with The Vroman effect [31].

Vroman's effect states that proteins with highest mobility in blood plasma , tend to arrive first and afterwards are replaced by less motile proteins with higher affinity to the surface. As per accepted notion and scientific studies the contact system is activated on negatively charged surfaces and that negative charge density in blood plasma controls the extent of the intensity of the contact activation in plasma confirming their positive corelation. In high chitosan samples the net surface charge is positive and it attracts blood cells to adhere to its surface frequently. Blood proteins are mostly negative and they tend to bind to sample surface as well. However fibrinogen amount in blood is comparatively lower than other blood protein like albumin. Thus at first the surface is flooded with abundant blood proteins. But fibrinogen has higher affinity towards platelet plug- negative charged surface and according to Vroman's effect they replace the higher motile proteins afterwards and

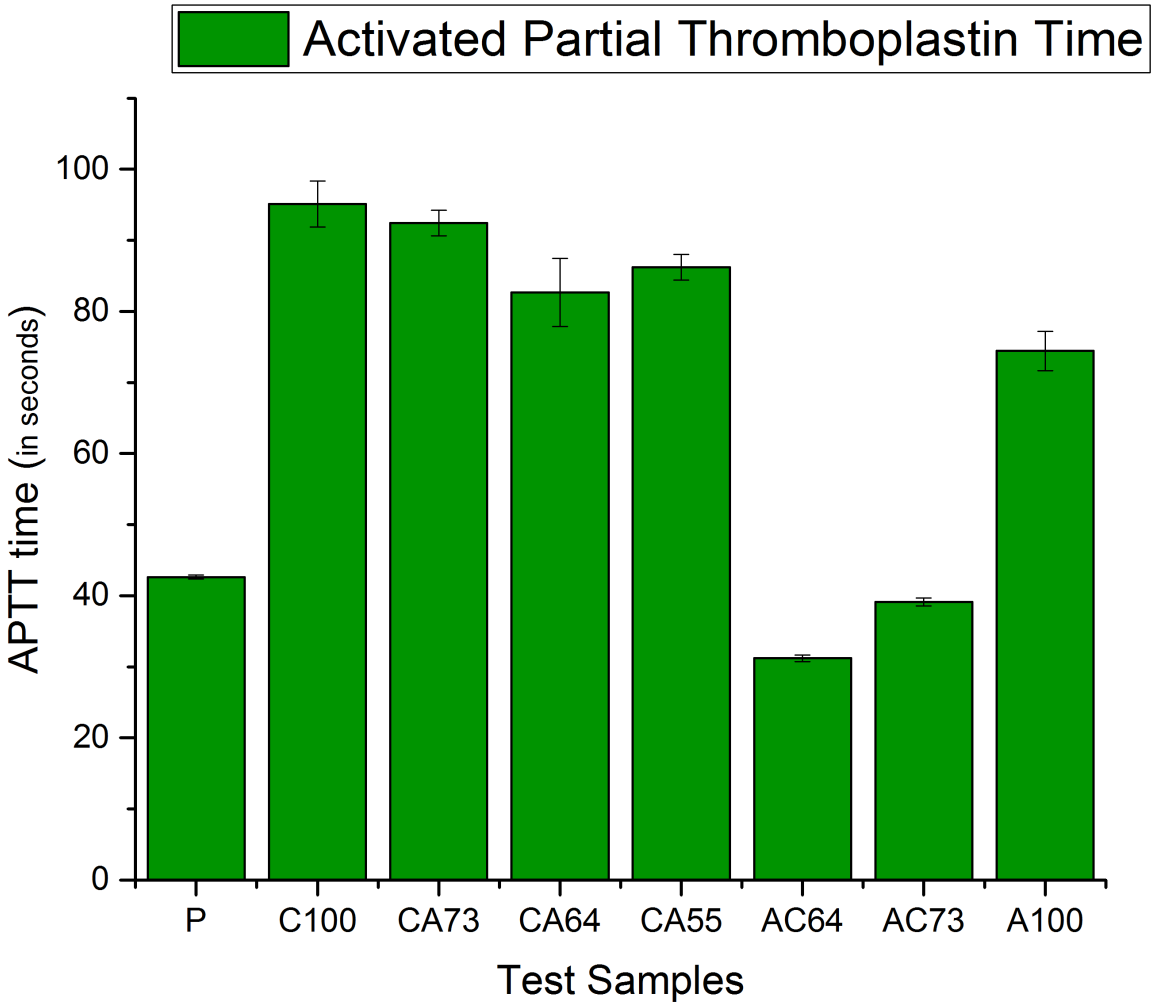


Figure 3.5: APTT time graph of PEC samples

initiate contact activation. This effect may be the explanation to the delayed aptt time in higher chitosan samples.

3.2.5 Platelet Adhesion

High cationic surface charge on chitosan surface increases its ability to promote platelet adhesion and activation which imparts to its procoagulant activity [32]. Activated platelets provide a dense negative charge phospholipid surface for fibrinogen adherence activating the platelets and thereafter the assembly of coagulation factor complexes and therefore accelerate blood coagulation.

Some studies indicated that besides adherent platelets, platelets in the bulk blood can also be activated when blood comes into contact with foreign materials.

Results indicate that pure chitosan showed the most platelet adherence and in PEC samples with greater net positive charge on surface, greater adherence followed. It may be due to the cationic surface effect of the the dressing samples. As shown, even higher alginate samples also showed considerable platelet adherence. This may be due to the tendency of

human blood cells to adhere to foreign materials in order to protect human body.

Platelets are cells without nucleus. When subjected to foreign surface or damaged endothelium, however they form clumps and produce pseudopodium and adhere to the surface [33]. The following FESEM image shows the adherence of platelets on a sample surface as clumps.

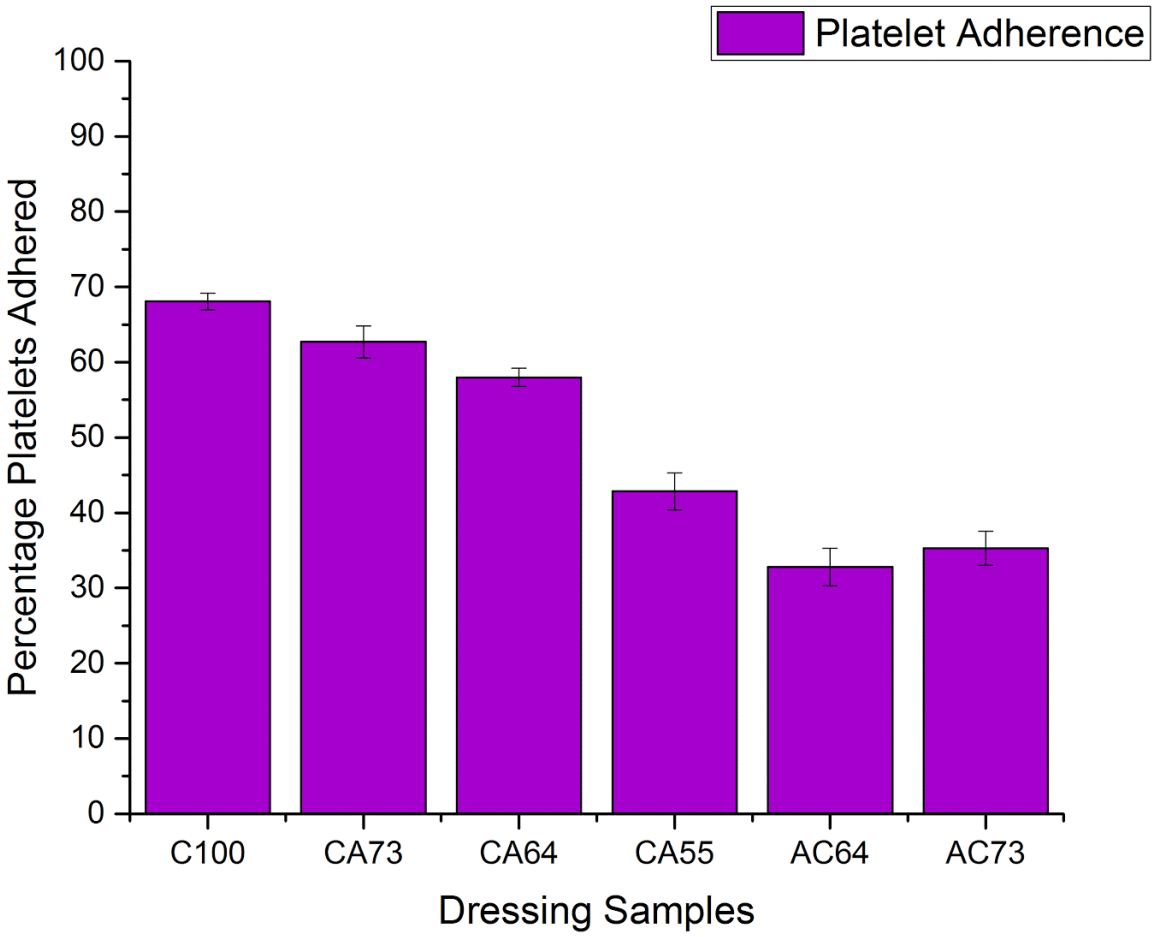


Figure 3.6: Percentage of platelets adhered in PEC samples

3.2.6 Swelling Studies

The following graph shows the swelling ratio of PECs with time.

The graph shows the swelling behaviour of sample C100, CA73, CA64, CA55. sample C100 got degraded on 3rd day however other PEC samples degraded on 4th day. C100 showed bes swelling ratio, highest being more than 50 times of its original weight. PEC samples showed little lower swelling ratio, which varied in and around 35-40 times of original weight. Sample CA64 started to lose water weight after first day itself. Below graph shows swelling studies of sample A100, AC64 and AC73. While performing the experiment, it was found that the sample A100 got dissolved in water instantly (within 10 minutes of start of the experiment.) However sample AC73 and sample AC64 retained their

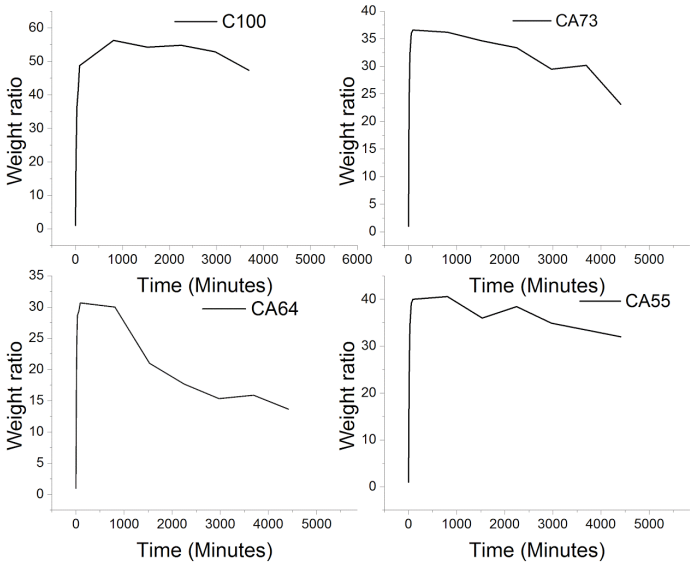


Figure 3.7: Swelling behaviour of C100, CA73, CA55 and CA64 (clock wise)

PEC structure for 2 days before degrading completely.

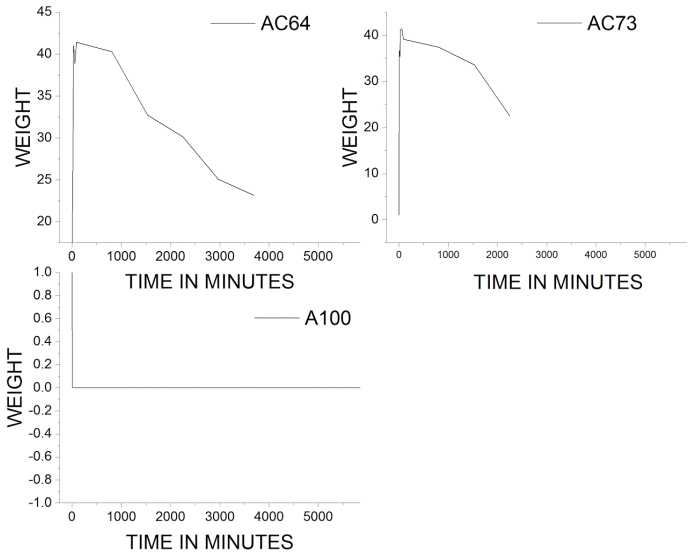


Figure 3.8: swelling behaviour of AC64, AC73 and A100 (clock wise)

Lower stability of high alginate PECs in PBS solution also questions , scaffold materials strength into question in case of deep wound.

3.3 Summary

From all the tests done on PEC samples, the results of hemolysis, platelet adhesion test and swelling studies were better for PECs with high chitosan concentration. In all clotting related tests like Prothrombin time, activated partial thromboplastin time it was found that positive charge of chitosan delayed the sample induced test results in PECs with high chitosan concentration. However the PEC samples with higher alginate proportions were found to have very little to no effect on test results. In blood clotting assay, pure chitosan and high chitosan concentration PECs showed delayed onset of clotting, though rate of clotting after onset was fast. From all these results Samples with higher chitosan concentration were chosen to be incorporated with clay mineral bentonite and further studies were carried out to evaluate the efficacy of the PEC clay nanocomposite samples.

Chapter 4

PEC-clay nanocomposite sample

4.1 Materials and Methods

Cell culture tasted Chitosan with degree of deacetylation greater than 90% and molecular biology grade sodium alginate were obtained from HiMedia. The test results were compared with HemCon ChitoGauze® PRO and Quikclot advanced clotting sponge. PT (Thromborel® S) and APTT (Actin FSL) reagents were obtained from Siemens Healthcare. Human thrombin-antithrombin complex (TAT) ELISA kit was obtained from Qayee-Bio.

4.1.1 Preparation of PEC Dressing

Chitosan solution (1% W/V) was prepared in water. Hydrochloric acid (HCl) was added dropwise to maintain acidic pH. Sodium alginate was dissolved in water. pH of chitosan solution was maintained around 5. Clay bentonite powder was added to chitosan solution and was stirred for 2 hours at high rotation for uniform mixing. Sodium alginate solution prepared has an acidic pH as well (pH 6-6.5). Chitosan-clay solution was taken in a syringe and dropwise added to alginate solution. Beads are formed in alginate base. Ultrasonification of these samples were done to prepare an uniform mixture which is the resultant PEC solution. The samples prepared were name S211, S311, S411 according to the chitosan , bentonite and alginate concentration respectively. These solutions were frozen at -200 C for 12 hours. Then the samples were lyophilised at -1200 C in a lyophilizer. Resultant scaffold produced is the desired material to be tested. Scaffolds of pure chitosan and alginate were also produced along wth simple PEC solutions. For characterisation study Environmental Scanning Electron Microscope (E-SEM), study was carried out.

4.1.2 Hemolysis Assay

Hemolysis assay was taken from Mallick et al. [1]. 0.9% NaCl solution was prepared by diluting common salt in water. This is known as physiological saline. Blood is diluted with physiological saline in the ratio of 4:5. The resulting solution forms the stock solution. The dressings were cut into equal sizes (12 mg each). The dressings were placed into

polypropylene tubes and 0.5 mL of diluted blood was dispensed on them. Two different control (positive and negative) were also made. Same amount of blood was added to both control, while .01M HCl was added to the positive control rupturing the RBC fraction. All the tubes were diluted with physiological saline by similar amount. The tubes were centrifuged at 2000g for 20 minutes. OD of the supernatants was taken at 540 nm.

$$Hemolysis\% = \frac{OD_{test} - OD_{-ve}}{OD_{+ve} - OD_{-ve}} \times 100 \tag{4.1}$$

Samples having hemolysis percentage <5% are highly hemocompatible, 5-20% are slightly hemolytic and samples having hemolysis percentage above 20% are non-hemocompatible.

4.1.3 Blood Clotting Assay

The whole blood clotting assay was taken from Shih et al. [2]. The dressings were placed into 50 mL polypropylene tubes and pre-warmed to 37ºC. 200 µL of anticoagulated whole blood was then dispensed onto the dressings. 20 µL of 0.2 M calcium chloride was added to initiate coagulation. After 10 min incubation at 37ºC and 30 rpm, the RBCs that were not trapped in the clot were hemolysed by pouring 25 mL distilled water slowly along the walls of the tube. This was done in an interval of 5, 10, 15, 20 minutes. The absorbance (OD) of the resulting hemoglobin solution was taken at 540 nm.

4.1.4 PT test

This test represents the duration in which the extrinsic pathway takes place. A prolonged PT time may signify an enhanced anticoagulant activity or chemical change to factor VII, X, V, II, I (fibrinogen). Another value INR is also associated with PT test. INR value greater than 1 shows delayed clotting. A typical range of INR for person not taking blood thinner medication is 2 to 3.5.

PT test of the sample dressing were done at CWS hospital Rourkela. Anticoagulated whole blood was collected and centrifuged at 4000g to isolate platelet poor plasma (PPP). Now 2 ml of PPP was taken in eppendorf tubes containing dressing material and was incubated at 37ºC for 1 hour.

100ml of sample treated PPP was transferred to test cup and was further incubated at 37ºC for 3 minutes and PT reagent was added 5 seconds prior to the end of incubation. PT reagent was also incubated and kept at 37ºC prior to use.

4.1.5 APTT test

APTT, also known as activated partial thromboplastin time is a medical test characterizing blood coagulation. The APTT test was performed at CWS hospital, Rourkela. Anticoagulated whole blood was collected and centrifuged at 4000g to isolate platelet poor plasma (PPP). Now 2 ml of PPP was taken in eppendorf tubes containing dressing material and was incubated at 37ºC for 1 hour. 100ml of sample treated PPP was transferred to test cup and was further incubated at 37ºC for 1 minute. 5 second prior to the incubation time finishes, APTT reagent was added and incubated for 3 more minutes. Again calcium chloride was added 5 seconds prior to the end of incubation.

4.1.6 Platelet Isolation and Adhesion

Whole blood was anticoagulated with trisodium citrate before collection. The anticoagulated whole blood was incubated at 37ºC for 15 minutes. Then the blood was collected in 15 ml polypropylene tubes and centrifuged at 800g for 15 minutes to separate blood cells and platelet rich plasma (PRP). About 2 ml of PRP was collected from 15 ml whole blood. The PRP is then mixed with PBS buffer at 1:3 ratio to increase the working volume. Now 100ml of the working solution was poured on the dressing. The dressing is now incubated for 15 minutes. After incubation, dressings were dip rinsed in PBS buffer thrice. This removes the unattached platelets from the dressings. To rupture the attached platelets, dressings were put inside PBS buffer containing 1% TritonX100 surfactant. The surfactant is used for LDH test to determine the percentage of platelets adhered to the dressings. A series of PRP with different dilution was treated with TritonX100 to obtain a platelet dilution curve and was compared to the sample data obtained to measure qualitatively the percentage of platelets adhered.

4.1.7 Thrombin Generation

Heparin, 15 U/mL, was used to collect whole blood. Heparinised whole blood was centrifuged at 1500g for 20 min. The plasma supernatant was separated from the RBC fraction. 1 mL of this plasma was poured over the dressings and incubated for 30 min at 37ºC for thrombin generation. The amount of antithrombin generated to neutralise the available thrombin in the dressings was determined using the amount of thrombin-antithrombin (TAT) complex produced with the help of ELISA kit according to manufacturer's instructions.

4.1.8 Swelling Studies

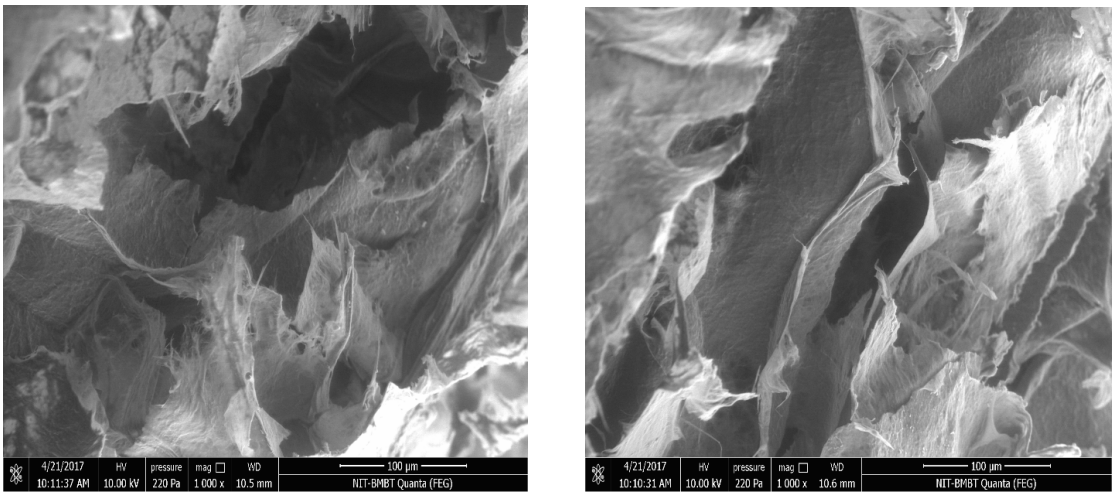
The swelling capacity of the dressings was determined in trisodium citrated whole blood and phosphate-buffered saline (PBS). The pre-weighed dry dressings (wini) were placed into tissue culture wells containing either trisodium citrated whole blood or PBS. The dressings

were weighed at an interval of 2 min for 30 min (Wwet). The swelling ratio of the dressings at each time interval was determined by the following formula:

$$Swellingratio = \frac{W_{wet} - W_{initial}}{W_{initial}} \times 100 \tag{4.2}$$

4.2 Results and Discussion

4.2.1 E-SEM



(a) (b)

Figure 4.1: E-SEM image of sample S211, S311

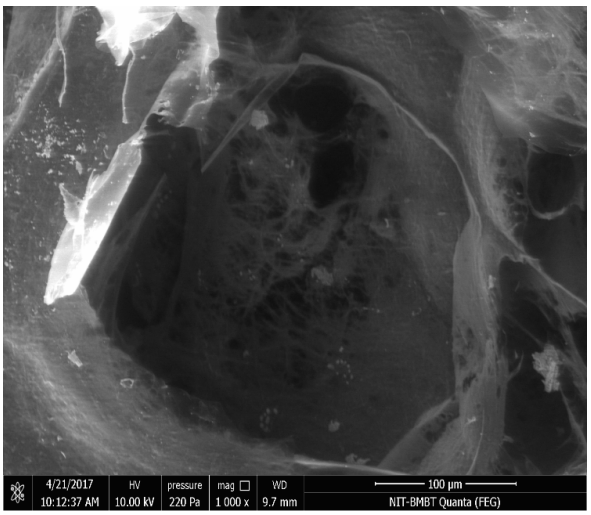


Figure 4.2: E-SEM image of sample S411

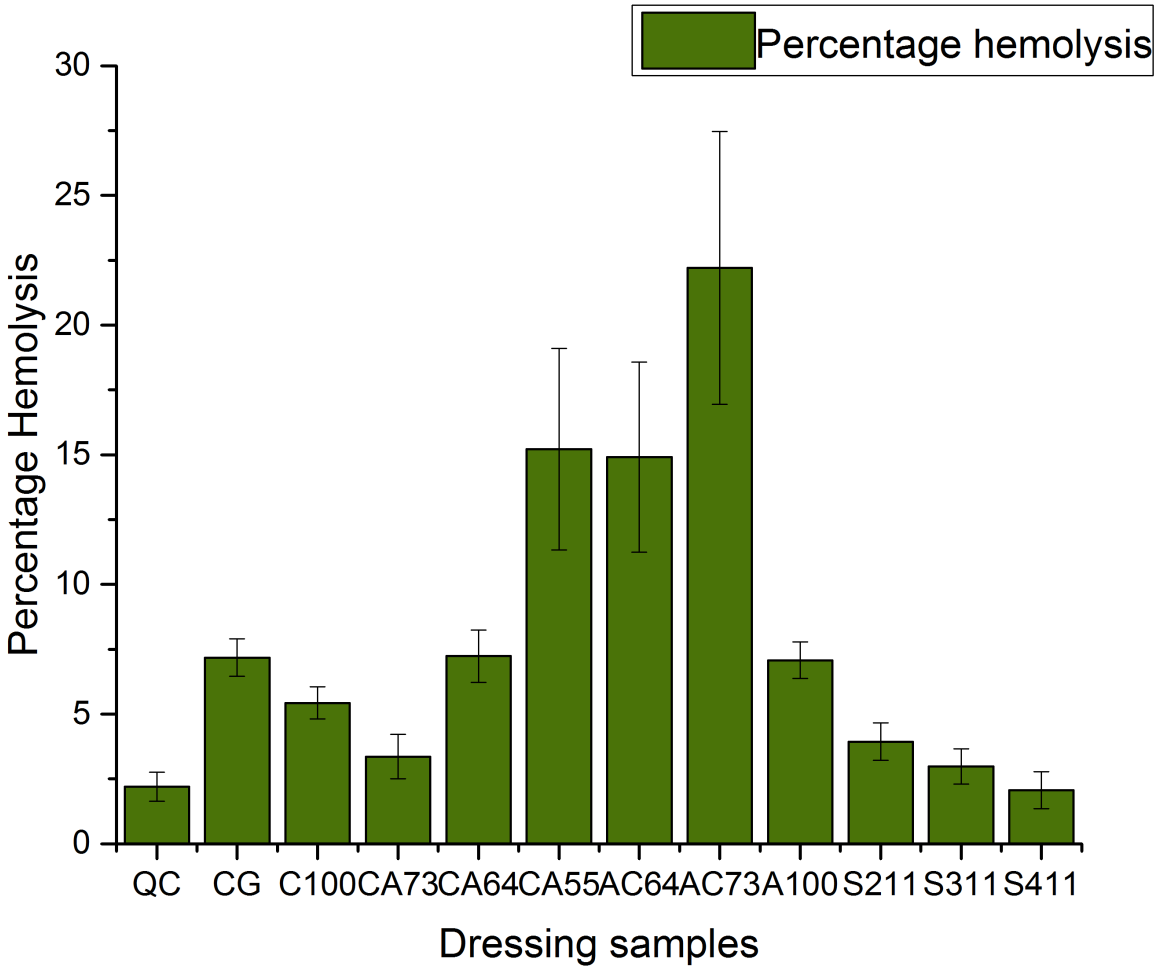


Figure 4.3: Hemolysis assay of nanocomposite samples

4.2.2 Hemolysis Assay

From the hemolysis study of bentonite incorporated samples, it is clear that inclusion of hemolysis on material doesn't have any hemolytic effect on blood erythrocytes, and percentage hemolysis was found to be less than 5% in case of all nanocomposite samples. Among the nanocomposites itself, the samples with higher cationic charge tend to show higher hemocompatibility. Comparing with the results obtained from Quickclot and Chitogauge, the samples are found to have competitive hemocompatibility characteristics.

4.2.3 Blood clotting assay

The following graph shows the clotting rate of nanocomposite samples. From the graph it is clear that sample S211 shows the best clotting rate. Incorporation of bentonite induces higher clotting rate.

Sample S411 shows the least clotting rate , even so it has comparable clotting rate to Quikclot. The slower clotting rate can be described by delay in activation of clotting cascade due to higher positive charge of the sample thereby causing Vroman's effect.

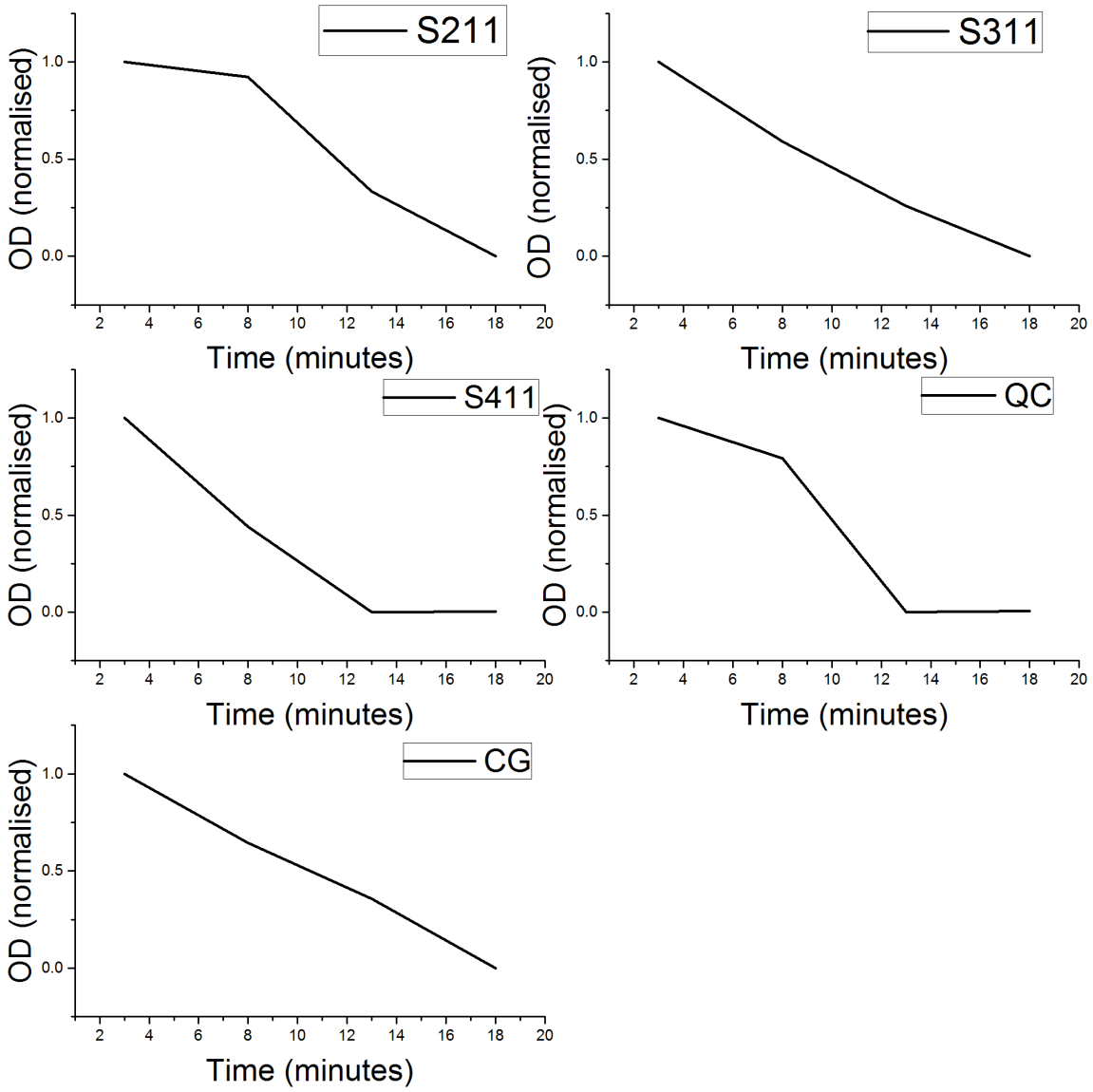


Figure 4.4: Blood clotting assay of nanocomposite samples

4.2.4 PT test

The following graph shows the time taken for the intrinsic pathway to get completed in nanocomposite samples which in turn depicts the sample induced variations in extrinsic pathway of blood clotting, if any.

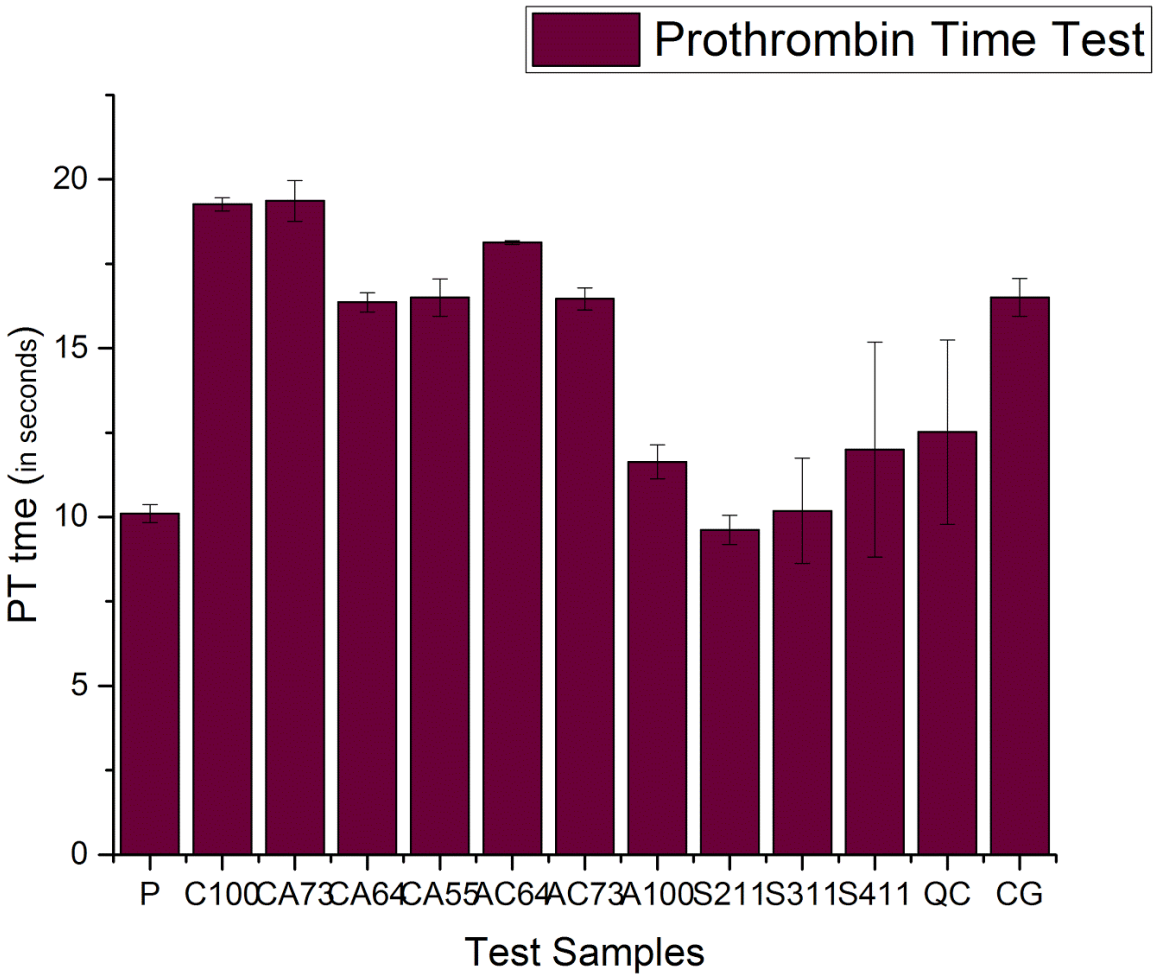


Figure 4.5: PT test of nanocomposite Samples

The graph compares nanocomposite samples with PEC samples and control samples i.e. Quikclot and Chitogauge. Bentonite incorporated samples showed the best result. The delay in PT time was least in sample S211, which was almost similar to normal blood plasma. In sample S411 , a little delay in PT time occurred but was lower than PEC samples and control samples as well. This shows that , Bentonite clay incorporation in PEC tends to improve PT time in dressing samples.

4.2.5 APTT Test

Similar to the PEC samples, from the aforementioned graph it is evident that surface positive charge has adverse effect on aptt time. The nanocomposite sample S211 , having the highest

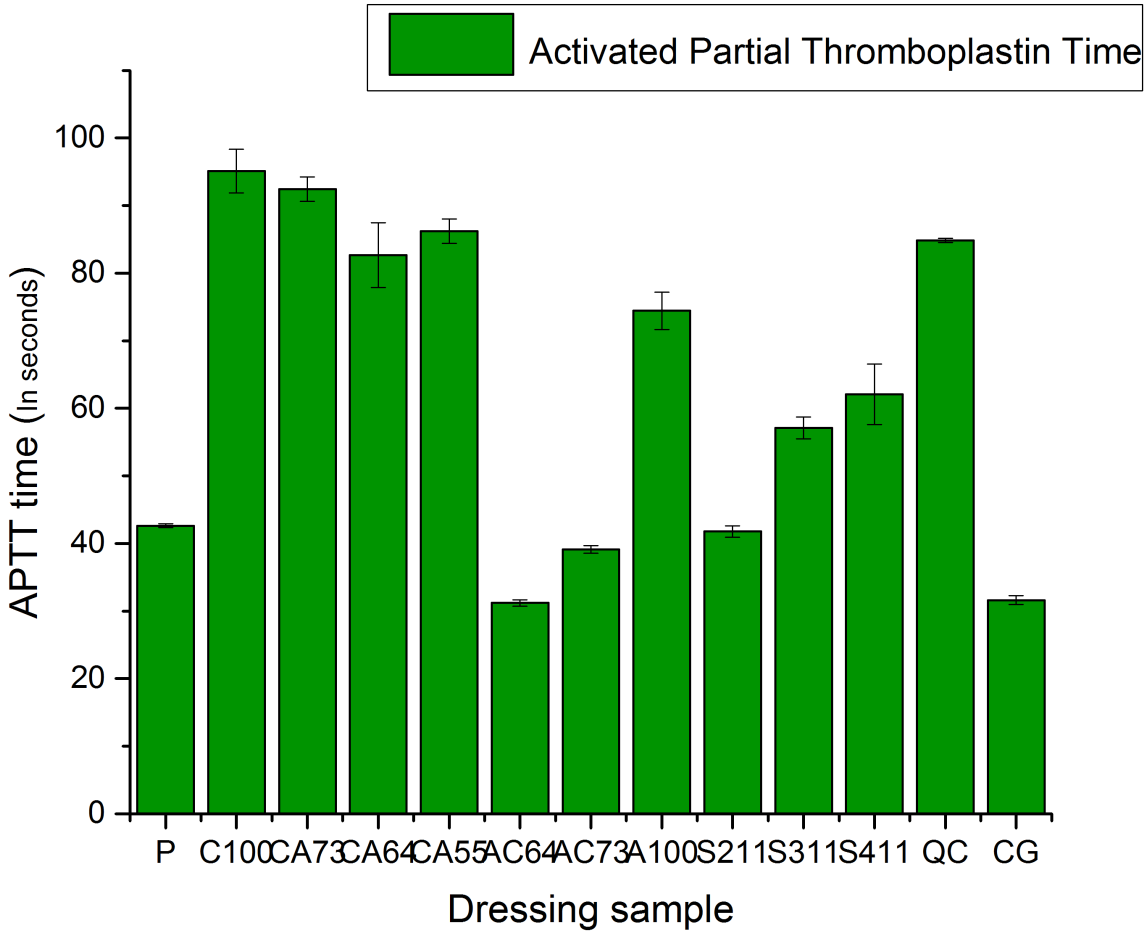


Figure 4.6: APTT test of nanocomposite samples

surface positive charge imparts a delay in aptt time. Sample S211 has almost no effect in normal intrinsic mechanism.

Among control samples, Quikclot showed a delayed aptt time. Chitogauge however has normal aptt time. The delayed time is explained by Vroman's effect, which shows no appreciable change or manipulation in the mechanism of intrinsic pathway but a little delay in contact activation which thereby delays the completion of the pathway. This shows that , Bentonite clay incorporation in PEC tends to improve PT time in dressing samples.

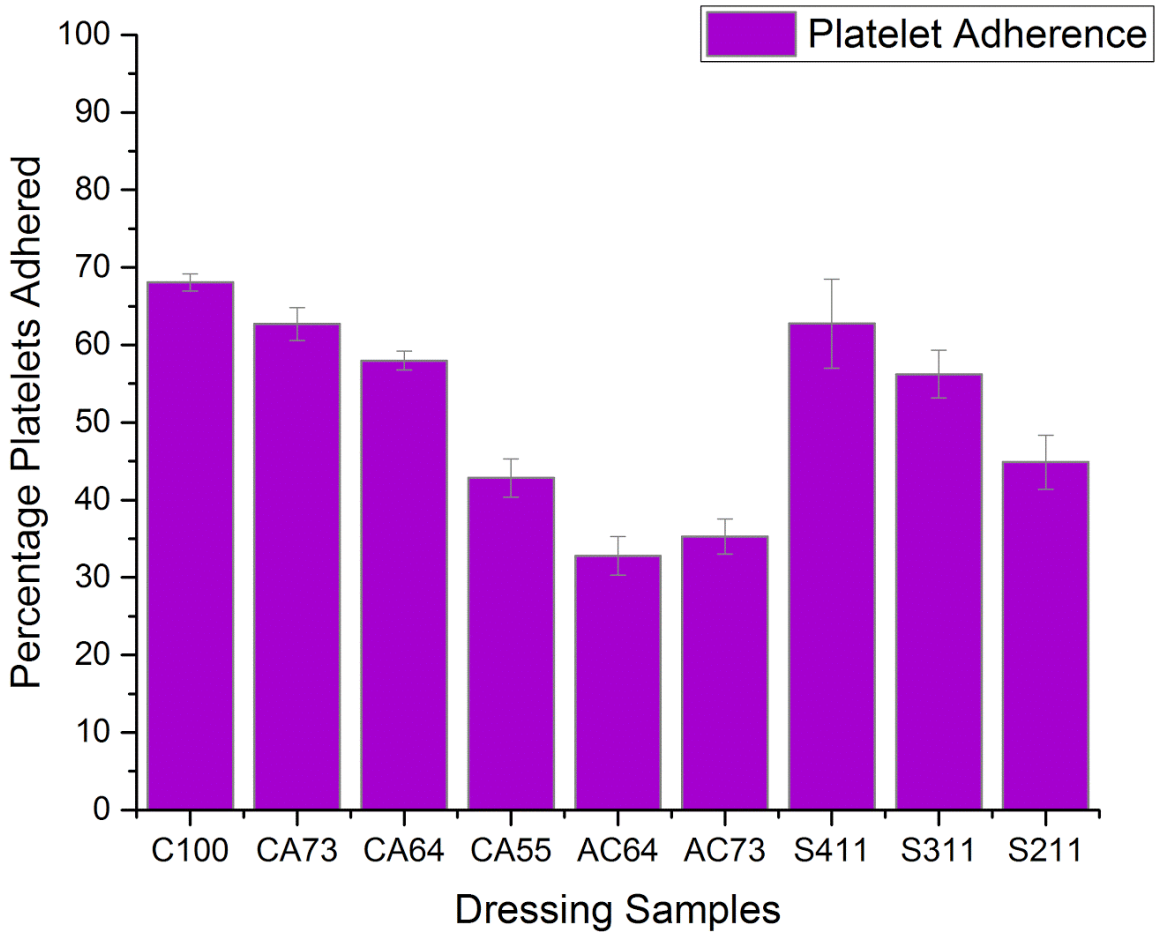


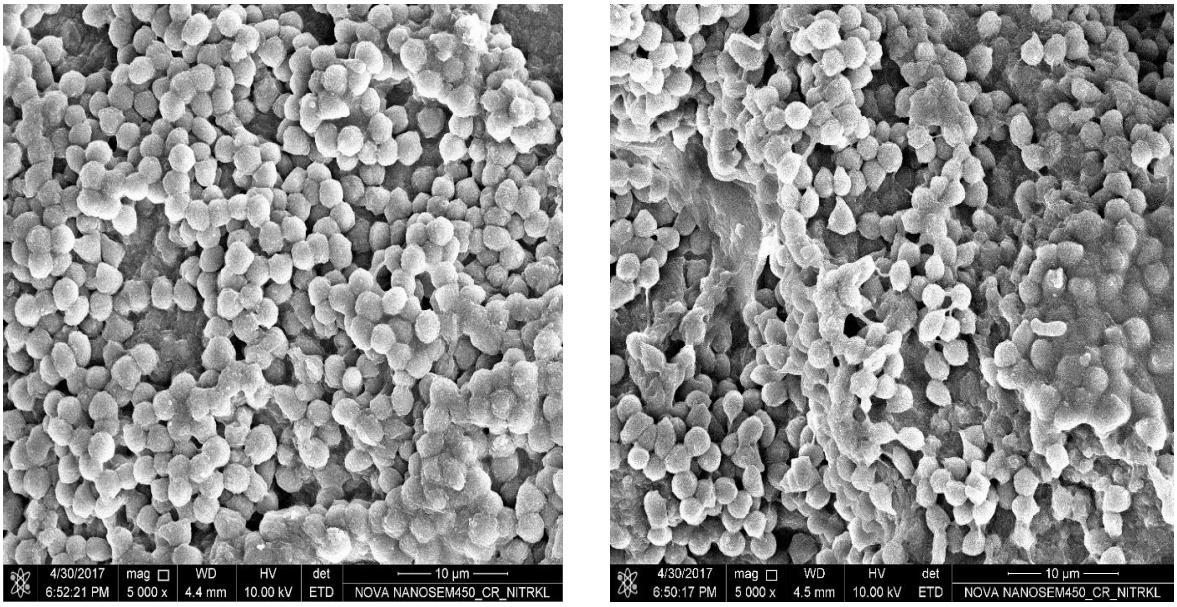
Figure 4.7: Percentage Platelet Adhesion on nanocomposite samples

4.2.6 Platelet Adhesion

As seen from the graph , nanocomposite sample S411 showed the better platelet adhesion and percentage platelet adhesion decreased with decrease chitosan concentration. In any case percentage of platelets adhered was above 30 percent and below 70%. As indicated in previous studies, platelets in the bulk blood can also be activated when blood comes into contact with foreign materials, which explains minimum 30% platelet adherence in all the samples despite net effective negative charge.

The following FESEM images show the platelet adherence to nanocomposite sample surface.

From the aforementioned FESEM images, platelets are seen to form clumps and are adhered to each other and sample surface as well, with pseudopodia.



(a) (b)

Figure 4.8: Platelet adherence on sample S211, S311

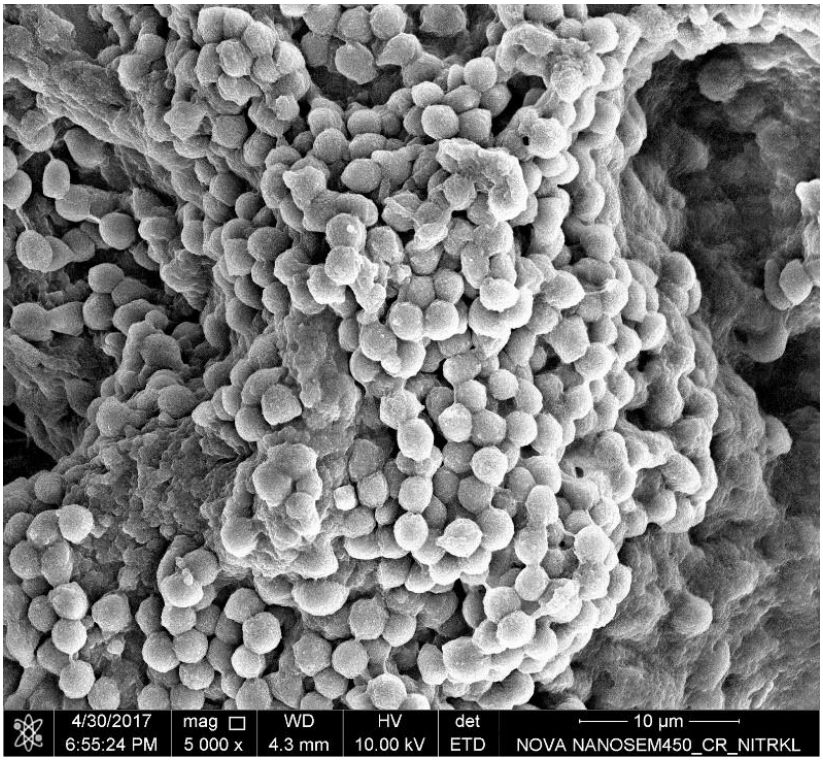


Figure 4.9: Platelet adherence on sample S411

4.2.7 Thrombin Generation

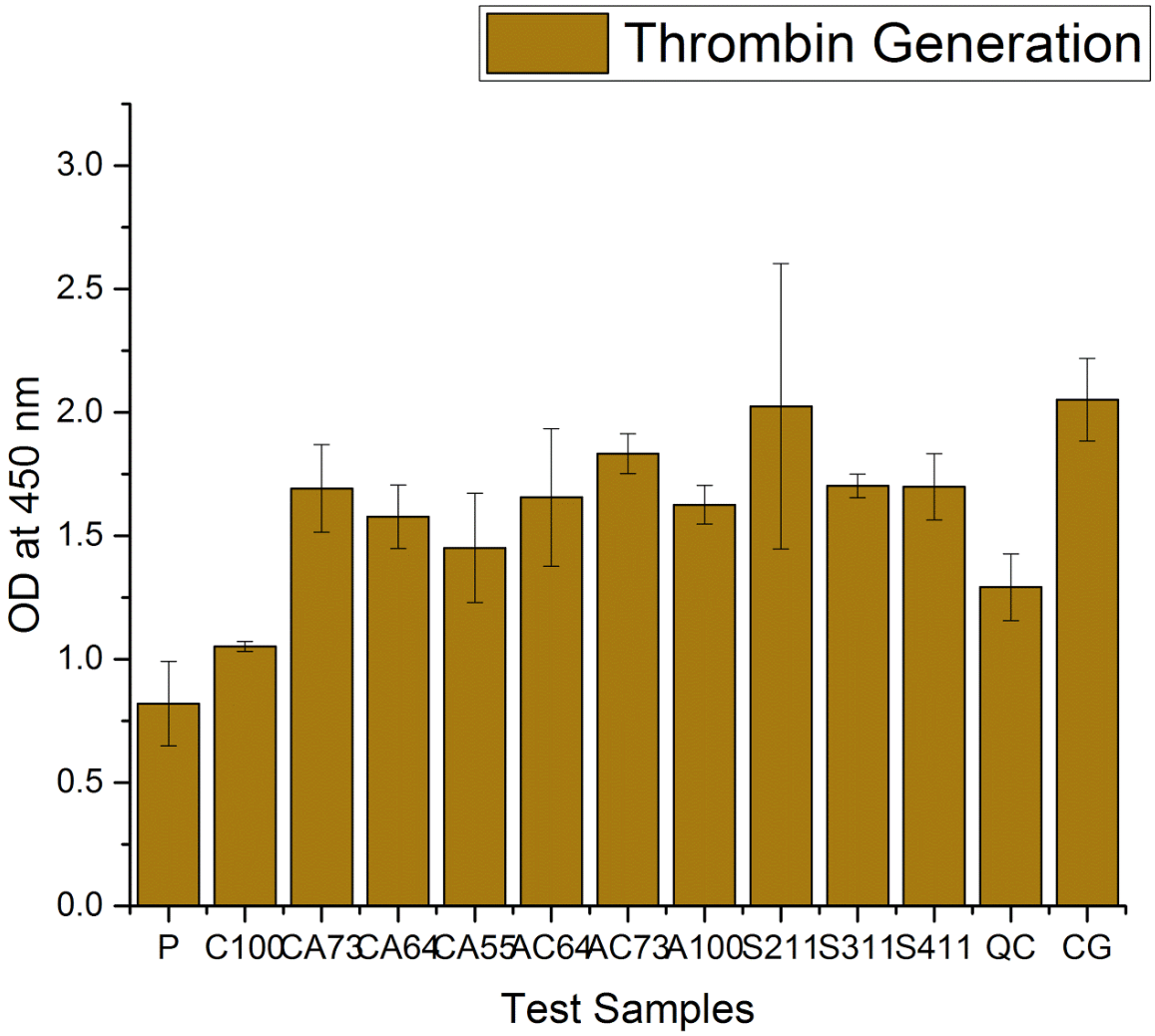


Figure 4.10: Thrombin generation graph of nanocomposite dressings

From the test samples, it is shown that all the samples promoted thrombin generation. Test sample chitogauge had the highest thrombin generation followed by sample S211, then AC73. Thrombin generation data is almost positively corelated with negative charge of sample. Among nanocomposite samples thrombin generation varied as S211 >S311 = S411.

4.2.8 Swelling studies

The following graph shows the swelling behaviour of the PEC-clay nanocomposite samples.

From the graph it is evident that all the Nanocomposite samples swelling ratio was lower than PEc samples. However their degradation didn't happen even until 5 days. Sample S211 maintained their saturated swelling state almost the entire time of the study. To verify the structural integrity of nanocomposite samples, sample dressings were dipped in blood

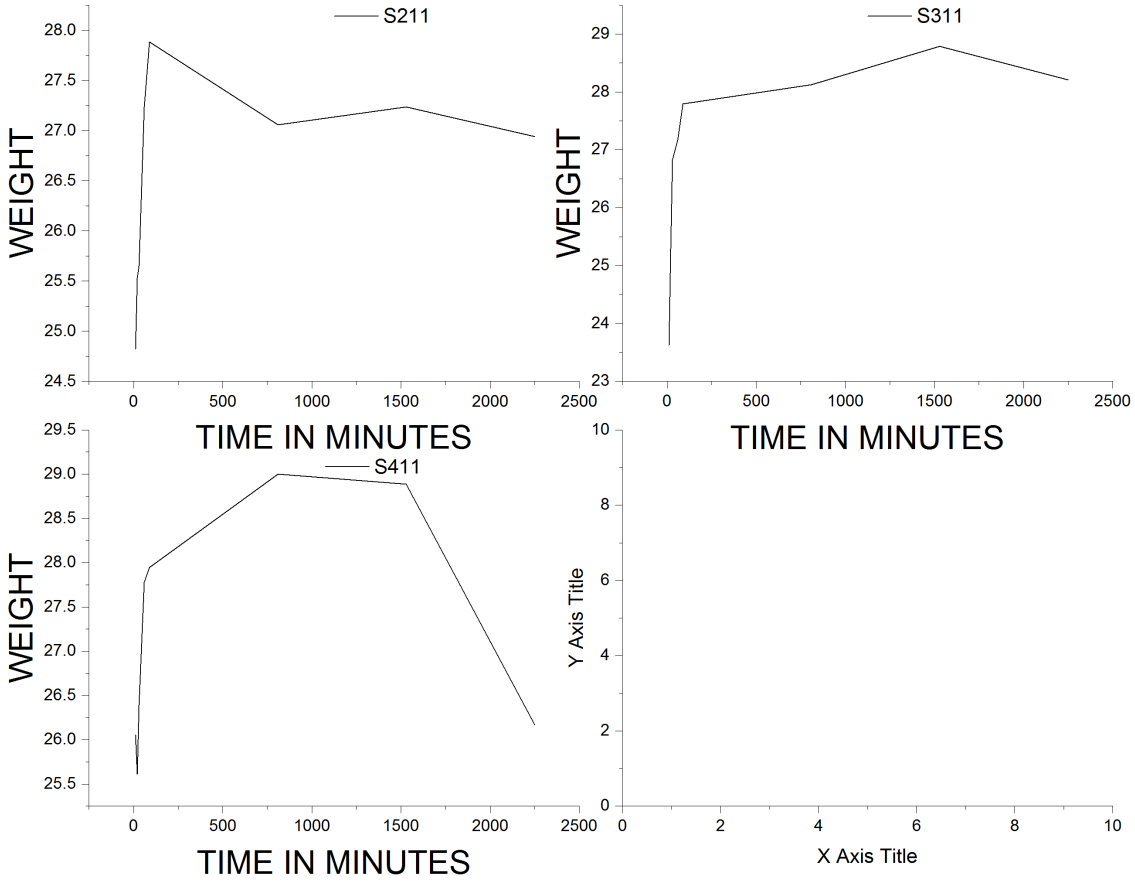
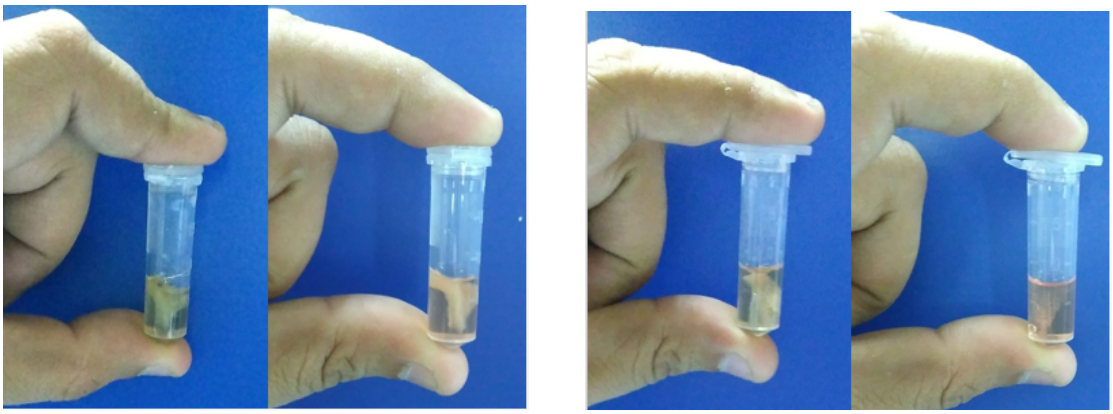


Figure 4.11: Swelling behaviour of S211, S311, S411 (clock wise)



(a) (b)

Figure 4.12: Image showing shape change in PEC clay sample compared to PEC sample

plasma for 6 hours and change in shape was observed. The same was compared with that of PEC structures. It was found that nanocomposite samples shape didn't change much , however PEC samples swelled heavily and change in shape was clearly visible.

Also from the swelling study it is known that PEC-clay nanocomposite samples initially absorbed water and attended peak value within 30 minutes. Even after 4 days they did not lose their integrity and carried almost same weight which was not the case of PEC samples. In alginate , sample dissolved and in chitosan too much swelling was observed. However chitosan changed its structure as it absorbed more plasma in the test. The structural integrity in nanocomposite samples may be explained by stable layered structure of clay mineral incorporated in PEC samples.

4.3 Summary

All the tests performed show that net positive charge on sample surface affected some test results. Sample S211 was better in case of PT and APTT results. However Sample S411 was better in case of platelet adhesion and hemolysis studies. Sample S211 also showed best clotting rate which was better than control materials as well.

Chapter 5

Conclusion

Induction of clay mineral into PEC dressing sample generates positive results in all tests. Results signify that net positive charge on dressing sample has adverse effect on clotting rate, due to Absorption dilution effect. However the higher the positive charge, the higher hemocompatibility and platelet adhesion.

However it is evident from these tests that bentonite is an excellent thrombogenic material. Its incorporation into PEC dressing yielded better results than simple PEC dressings. Future studies on the material with varied concentration can result in a product with optimum results in all tests which can be best effective as wound dressing material.

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